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NEWS 22	FEB 28	REGISTRY/ZREGISTRY enhanced with more experimental spectral property data
NEWS 23	MAR 01	INSPEC reloaded and enhanced
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FILE 'HOME' ENTERED AT 11:03:53 ON 16 MAR 2006

=> file medline

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.21	0.21

FILE 'MEDLINE' ENTERED AT 11:04:08 ON 16 MAR 2006

FILE LAST UPDATED: 15 MAR 2006 (20060315/UP). FILE COVERS 1950 TO DATE.

On December 11, 2005, the 2006 MeSH terms were loaded.

The MEDLINE reload for 2006 is now (26 Feb.) available. For details on the 2006 reload, enter HELP RLOAD at an arrow prompt (=>). See also:

http://www.nlm.nih.gov/mesh/
http://www.nlm.nih.gov/pubs/techbull/nd04/nd04_mesh.html
http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_med_data_changes.html
http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_2006_MeSH.html

OLDMEDLINE is covered back to 1950.

MEDLINE thesauri in the /CN, /CT, and /MN fields incorporate the MeSH 2006 vocabulary.

This file contains CAS Registry Numbers for easy and accurate substance identification.

=> e benjamin/au

E1	4		BENJAMIM CLAUDIA F/AU
E2	2		BENJAMIM CLAUDIA FARIAS/AU
E3	0	-->	BENJAMIN/AU
E4	49		BENJAMIN A/AU
E5	3		BENJAMIN A B/AU
E6	2		BENJAMIN A C/AU
E7	1		BENJAMIN A C W/AU
E8	4		BENJAMIN A D/AU
E9	27		BENJAMIN A E/AU
E10	2		BENJAMIN A E JR/AU
E11	1		BENJAMIN A F/AU
E12	1		BENJAMIN A G/AU

=> e

E13	1	BENJAMIN A H/AU
E14	1	BENJAMIN A I/AU
E15	4	BENJAMIN A L/AU
E16	19	BENJAMIN A M/AU
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E2            1      BENJAMIN COMPTON/AU
E3          178 --> BENJAMIN D/AU
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E24           2      BENJAMIN DENIS R/AU

=> s e5
L1           71 "BENJAMIN D C"/AU

=> s bovine serum albumin
        139869 BOVINE
        581928 SERUM
        102813 ALBUMIN
L2       14976 BOVINE SERUM ALBUMIN
                 (BOVINE(W)SERUM(W)ALBUMIN)

=> s l2 and l1
L3           10 L2 AND L1

=> d ti 1-10

L3   ANSWER 1 OF 10     MEDLINE on STN
TI   Monoclonal antibodies to bovine serum albumin
     : affinity and specificity determinations.

L3   ANSWER 2 OF 10     MEDLINE on STN
TI   Immune responses to complex protein antigens I. MHC control of immune
     responses to bovine albumin.

L3   ANSWER 3 OF 10     MEDLINE on STN
TI   The antigenic structure of bovine serum
     albumin. Evidence for multiple, different, domain-specific
     antigenic determinants.

L3   ANSWER 4 OF 10     MEDLINE on STN
TI   Antibody as an immunological probe for studying the refolding of
     bovine serum albumin. An immunochemical
     approach to the identification of possible nucleation sites.

L3   ANSWER 5 OF 10     MEDLINE on STN
TI   Antibody as immunological probe for studying refolding of bovine
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serum albumin. Refolding within each domain.

L3 ANSWER 6 OF 10 MEDLINE on STN
TI Antibody as an immunological probe for studying the refolding of **bovine serum albumin**. II. Evidence for the independent refolding of the domains of the molecule.

L3 ANSWER 7 OF 10 MEDLINE on STN
TI Antibody as an immunological probe for studying the refolding of **bovine serum albumin**. I. The catalysis of reoxidation of reduced **bovine serum albumin** by glutathione and a disulfide interchange enzyme.

L3 ANSWER 8 OF 10 MEDLINE on STN
TI The termination of immunologic unresponsiveness to the cyanogen bromide fragments of **bovine serum albumin** in rabbits.

L3 ANSWER 9 OF 10 MEDLINE on STN
TI The termination of immunological unresponsiveness to **bovine serum albumin** in rabbits. 3. Structural and serological relationships among various serum albumins and their cyanogen bromide fragments.

L3 ANSWER 10 OF 10 MEDLINE on STN
TI The termination of immunological unresponsiveness to **bovine serum albumin** in rabbits. I. Quantitative and qualitative response to cross-reacting albumins.

=> d 4

L3 ANSWER 4 OF 10 MEDLINE on STN
AN 79048421 MEDLINE
DN PubMed ID: 81833
TI Antibody as an immunological probe for studying the refolding of **bovine serum albumin**. An immunochemical approach to the identification of possible nucleation sites.
AU Chavez L G Jr; **Benjamin D C**
SO The Journal of biological chemistry, (1978 Nov 25) Vol. 253, No. 22, pp. 8081-6.
Journal code: 2985121R. ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197901
ED Entered STN: 19900314
Last Updated on STN: 19970203
Entered Medline: 19790124

=> d 3

L3 ANSWER 3 OF 10 MEDLINE on STN
AN 79048422 MEDLINE
DN PubMed ID: 81834
TI The antigenic structure of **bovine serum albumin**. Evidence for multiple, different, domain-specific antigenic determinants.
AU **Benjamin D C**; Teale J M
SO The Journal of biological chemistry, (1978 Nov 25) Vol. 253, No. 22, pp. 8087-92.
Journal code: 2985121R. ISSN: 0021-9258.

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CY   United States
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   197901
ED   Entered STN: 19900314
     Last Updated on STN: 19970203
     Entered Medline: 19790124


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E8            1     WOODWARD M E/AU
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E10           6     WOODWARD M G/AU
E11           1     WOODWARD M H/AU
E12           1     WOODWARD M I/AU

=> e
E13         106     WOODWARD M J/AU
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E24           1     WOODWARD MELANIE J/AU

=>
=> e
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E26           5     WOODWARD MICHAEL/AU
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E32           4     WOODWARD N J/AU
E33           1     WOODWARD N M/AU
E34           4     WOODWARD N W/AU
E35           2     WOODWARD N W JR/AU
E36           1     WOODWARD N W SR/AU

=> s e16 or e26
            10 "WOODWARD M P"/AU
             5 "WOODWARD MICHAEL"/AU
L4          15 "WOODWARD M P"/AU OR "WOODWARD MICHAEL"/AU

=> s l4 and l3
L5           0 L4 AND L3

=> d his

     (FILE 'HOME' ENTERED AT 11:03:53 ON 16 MAR 2006)
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     FILE 'MEDLINE' ENTERED AT 11:04:08 ON 16 MAR 2006
                E BENJAMIN/AU
                E BENJAMIN D/AU
L1             71 S E5
L2          14976 S BOVINE SERUM ALBUMIN
L3             10 S L2 AND L1
                E WOODWARD M/AU
L4             15 S E16 OR E26
L5              0 S L4 AND L3

=> s l1 and l4
L6             2 L1 AND L4

=> d 1,2

L6   ANSWER 1 OF 2     MEDLINE on STN
AN   91123698     MEDLINE
DN   PubMed ID: 1704035
TI   The antigenic surface of staphylococcal nuclease. I. Mapping epitopes by
     site-directed mutagenesis.
AU   Smith A M; Woodward M P; Hershey C W; Hershey E D; Benjamin
     D C
CS   Department of Microbiology, School of Medicine, University of Virginia,
     Charlottesville 22908.
NC   5-SO7-RR05431 (NCRR)
     5T32GM08136 (NIGMS)
     AI-20745 (NIAID)
SO   Journal of immunology (Baltimore, Md. : 1950), (1991 Feb 15) Vol. 146, No.
     4, pp. 1254-8.
     Journal code: 2985117R. ISSN: 0022-1767.
CY   United States
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Abridged Index Medicus Journals; Priority Journals
EM   199103
ED   Entered STN: 19910405
     Last Updated on STN: 19960129
     Entered Medline: 19910308

L6   ANSWER 2 OF 2     MEDLINE on STN
AN   87192764     MEDLINE
DN   PubMed ID: 2437011
TI   A unique epitope on human serum albumin recognized by monoclonal antibody
     HSA-1: a probe for identification of the human origin of blood or tissue.
AU   Benjamin D C; Herr J C; Sutherland W M; Woodward M P;
     DeCourcy K; Condon T P
SO   Hybridoma, (1987 Apr) Vol. 6, No. 2, pp. 183-90.
     Journal code: 8202424. ISSN: 0272-457X.
CY   United States
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   198705
ED   Entered STN: 19900303
     Last Updated on STN: 19900303
     Entered Medline: 19870527
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Ann. Rev. Immunol. 1984. 2:67–101
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SRNT

THE ANTIGENIC STRUCTURE OF PROTEINS: A REAPPRAISAL

David C. Benjamin[1], *Jay A. Berzofsky*[2], *Iain J. East*[3], *Frank R. N. Gurd*[4], *Charles Hannum*[5], *Sydney J. Leach*[6], *Emanuel Margoliash*[7], *J. Gabriel Michael*[8], *Alexander Miller*[9], *Ellen M. Prager*[10], *Morris Reichlin*[11], *Eli E. Sercarz*[9], *Sandra J. Smith-Gill*[12], *Pam E. Todd*[6], *and A.C. Wilson*[10]

INTRODUCTION

Proteins are one of the most abundant and diverse classes of antigens to which the immune system can respond. These include transplantation antigens, antigens of infectious and parasitic organisms, and allergens. An understanding of host defense mechanisms and of the ability to distinguish self from nonself requires a knowledge of the structural basis for protein antigenicity. The advent of hybridoma technology (1) to produce monoclonal antibodies, each of which

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0732-0582/84/0610-0067$02.00

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binds to a specific area on the protein surface known as an antigenic site, has provided a powerful new tool for investigating antigen structure. Recent studies from a number of laboratories using monoclonal antibodies directed toward structurally defined protein antigens have produced a large body of new information which necessitates a reevaluation of the nature and distribution of the antigenic sites on proteins. Although in this review we emphasize those antigenic sites [antigenic determinants or epitopes (2), terms used interchangeably] recognized by antibodies, we also include limited recent data on the nature of the sites recognized by T cells.

Immunogenicity indicates the ability to elicit an immune response and is the summation of a variety of influences that reflect the previous history of the animal as well as its genetic attributes: the available B-cell repertoire, the activity of T-helper cells and T-suppressor cells, the idiotype network, and the major histocompatibility complex (MHC). In contrast, *antigenicity* merely implies the ability to be recognized by the product of the immune response, i.e. antibodies or immune cells. As has recently been emphasized (3), chemically synthesized peptides may elicit antibodies reactive with determinants on the native protein. The present discussion, however, is limited to studies where antibodies were elicited by immunization with the native protein. While peptides or other derivatives may have been used in such studies to identify antigenic sites reactive with antibody, the sites under discussion are those that are immunogenic on the native molecule.

The fact that antibodies elicited by a native protein often do not react with the denatured form (4,5) and that specific antibodies can be raised against peptides of undefined conformation (3,5–9) led to the definition of two classes of antigenic determinants. *Conformational determinants* were defined as dependent on the native spatial conformation of the protein, while *sequential determinants* were defined as depending only on the amino acid sequence of the corresponding peptide segment (9,10). This operational distinction does not imply different antigenic binding mechanisms. A "conformational" determinant on a native protein can usually be inhibited from interacting with its antibodies by a large molar excess of the corresponding "sequential" peptide. The native conformation is one of many conformations that such a peptide fragment can assume in solution (9–13). *All* determinants are now seen to be conformational in the sense that the antibody combining sites will bind with a measurable affinity only to that population of antigen conformers which presents a complementary constellation of interacting side chains. It follows that antigenic determinants are topographic, i.e. composed of structures on the protein surface. Topographic determinants may be contained within a single *segment* of the amino acid sequence (but not necessarily involving all contiguous residues in the segment), or *assembled* from residues far apart in the amino acid sequence but brought together on the surface by the folding of

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the protein in its native conformation. This latter category probably represents the major group of conformational determinants. Of particular importance in discussions of antigenicity in proteins are the amino acid sequence differences among evolutionary variants of a protein. Such differences often have little or no detectable effect on the backbone conformation but can produce antigenically crucial changes in the nature of the protein surface (14–16).

It must be emphasized that the set of specificities that predominate after immunization with the native protein is clearly not equal to the total potential repertoire. Certain sequences or even individual residues on the surface of proteins have been identified as "immunodominant" sites, i.e. those to which most of the immune response is directed. Several mechanisms could explain immunodominance. One view is that special structural properties intrinsic to certain regions of a protein make these regions immunodominant. An alternative view is that immunodominance of a given region depends on the regulatory mechanisms of the host, including tolerance to structures resembling self, immune response genes, the specificity of T-cell help, and idiotype networks.

Many questions remain, and we explore some in this review. Can all surface structures on a protein molecule be recognized by the immune system as determinants? Are there sites that are intrinsically immunogenic, independent of the responding species? How many of the potential determinants are recognized by an individual's B and T cells? To what extent do homologous proteins in the responding individual affect the expressed repertoire? What is the relationship between the expressed repertoires of B and T cells? How does the expressed T-cell repertoire regulate the B-cell repertoire?

The studies described below have used four structurally well-characterized globular proteins—myoglobin, lysozyme *c*, cytochrome *c*, and serum albumin—as model antigens. Although some different approaches were employed in the study of each protein, several major common conclusions emerge. First, most, if not all, of the surface of a protein may be immunogenic and antigenic and may include multiple, overlapping determinants. Second, most antigenic sites consist of a three-dimensional array of amino acid residues that require the native conformation of the protein for their antigenic integrity. Third, on a given antigen the subset of potential determinants that are immunogenic varies from species to species and depends on the structural differences between that antigen and the host's self proteins, and on regulatory mechanisms that govern interactions among the many subpopulations of cells generating the immune response.

MYOGLOBIN

Sperm whale myoglobin, 153 amino acid residues long, was one of the first proteins to be sequenced (17) and to have its three-dimensional structure deter-

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mined by X-ray crystallography (18,19). Therefore, it was also one of the first to be chosen as a model antigen. Crumpton & Wilkinson (20) first localized some of the antigenic sites using proteolytic fragments of myoglobin to inhibit quantitative precipitin reactions. For rabbit antibodies to sperm whale myoglobin, peptides consisting of residues 15–29 and 147–153 inhibited most strongly. Three other peptide fragments, 56–69, 70–76, and 139–146, gave less inhibition or less consistent inhibition with different antisera. Subsequently, Atassi (21) described "the complete immunochemical anatomy" of myoglobin as consisting of five antigenic sites. Each of these sites, residues 15–22, 56–62, 94–99, 113–119, and 145–151, is composed of six to eight consecutive residues at a bend or exposed corner in the native protein. Sites I and V are contained in the two major sites observed by Crumpton & Wilkinson (20), and these were also observed by a combined solid-phase peptide synthetic and immunoassay procedure in Leach's laboratory (22,23). Site II is contained in the peptide 56–69 found to be weakly antigenic with some antisera by Crumpton & Wilkinson. Besides describing the two new sites III and IV (21), Atassi reported that 99.8% of the antibodies in various antisera could be removed by binding sequentially to these five synthetic sites (24). Thus, he postulated that these sites constituted the only antigenic determinants on sperm whale myoglobin (21,24,25) and that they were the only antigenic sites on myoglobins from any mammalian species, regardless of the amino acid substitutions at these sites (24,25). It is true that spatially homologous regions in a soybean leghemoglobin molecule are immunogenic in spite of the great evolutionary distance between mammalian and plant globins (26). However, the delineation of antigenic sites using small peptides must be reconsidered in the light of the recent findings on general charge and hydrophobic effects described below. Twining et al (25) also postulated that the antibody response to myoglobin is independent of the immunized species, in conflict with earlier views on the role of the responding species (14).

Although the characterization of five antigenic sites of myoglobin constituted a historic step in describing the immunochemical anatomy of a protein, results from several laboratories published in the last few years have produced a new and rather different view of the antigenic structure of myoglobin. These recent results disprove some of the earlier generalizations cited above and lead to a very different concept of protein antigenicity.

1. One of the most important findings is the existence of a number of antigenic sites of myoglobin that are not contained within a single segment of a peptide chain but are *assembled* from several segments. Such sites require the native conformation for their integrity and may not exist in complete form in any single cleavage fragment of the protein. Some specific antibodies will bind peptides containing continuous sequences, albeit with lower affinities than they bind the native structure (27), either because the peptides lack the

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native conformation or because they form only part of a topographic site, or both. The degree to which the affinity is lowered depends on the size and the conformational equilibrium of the peptide (27). However, binding of a peptide to antibodies will siphon the equilibrium over toward the native form, and so the antibodies can still be trapped on an immunoadsorbent carrying the peptide fragment.

Early evidence for assembled topographic sites came from a study of the cross-reactivities of different myoglobins for the fraction of antibodies to beef myoglobin specific for peptide 1–55 of the molecule (28). These antibodies bind with markedly different affinities to beef, sheep, and pig myoglobins in spite of their identity in the 15–22 sequence. These results imply either the involvement of other residues outside of residues 15–22 within this determinant or the existence of another determinant within the 1–55 fragment. Other evidence for the presence of assembled topographic antigenic sites came from Lando et al (29), who found that of the antibodies in each of four antisera to sperm whale myoglobin (from three different species) that bound with high affinity to the native molecule, 30–40% failed to bind to affinity columns of any of the three CNBr (cyanogen bromide) cleavage fragments that make up the whole protein. None of these results could be explained by the existence of five discrete sequential antigenic sites reported earlier (21).

Independent evidence for assembled topographic antigenic sites on myoglobin and of their high frequency of occurrence came from studies of mouse monoclonal antibodies to sperm whale myoglobin, none of which bound to the amino-terminal or carboxy-terminal CNBr fragments (30). Six of those with high affinity (10^8 to 10^9 M^{-1}) (30) were studied in detail, and none bound to any of the three CNBr fragments spanning the whole sequence of the molecule (31). That is, none bound to sequential sites, including sites I–V above. Assignments of antigenic sites could be made for three of these monoclonal antibodies by comparing their relative affinities for 13 to 15 different myoglobins with the sequences of these myoglobins (31). One monoclonal antibody reacted with a site that included Lys 140, a second with a site involving Glu 4, Lys 79, and possibly His 12, and a third with a site involving Glu 83, Ala 144, and Lys 145 (31). The last two sites are clearly assembled topographic sites, in that they include residues far apart in the primary sequence but brought together on the surface of the molecule by the way it folds in the native conformation (Figure 1A,B). Moreover, neither of these sites exists on any single CNBr fragment.

Additional evidence for assembled topographic sites comes from a study of two monoclonal antibodies to human myoglobin (33). Based on comparison of binding to different myoglobins, one monoclonal antibody was found to recognize a site that included residues 34 and 113 or possibly 34 and 53 and the second a site that included residues 74, 87, and 142. Again, these residues

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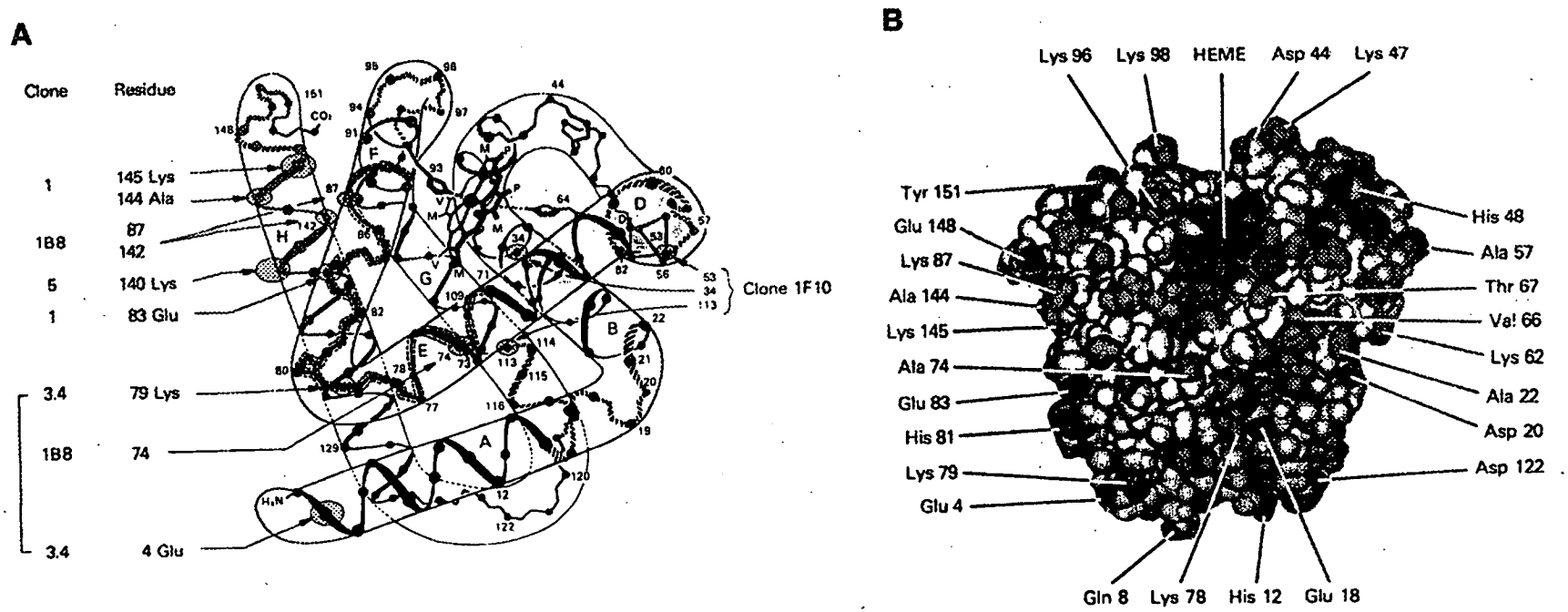

Figure 1 *Panel A:* Line drawing representing the three-dimensional structure of the alpha-carbon backbone of native sperm whale myoglobin [modified from (32) with permission]. The alpha helixes are labeled A through H (the C helix is partly hidden by the heme). Side chains are omitted except for two histidine rings (64 and 93) involved with the heme. The letters P, M, V on the heme indicate the propionic, methyl, and vinyl groups, respectively, of the porphyrin ring. Residues 1 and 2 at the amino-terminus are not shown. Residues recognized by monoclonal antibodies to sperm whale myoglobin (clones 1, 3.4, and 5) (31) and to human myoglobin (clones 1B8 and 1F10) (33) are indicated. Five reported "sequential" sites (21) are indicated by crosshatching, and a sixth region of sequence to which antibodies have been found to bind (residues 72–88) (34) is shown by dashed outlining.

Panel B: Computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano (19) X-ray diffraction coordinates. This orientation is the same as that in Panel A. The computer method was described by Feldmann et al (35). The carboxyl oxygens are shaded darkest, followed by the heme and aromatic carbons, aliphatic side-chain carbons, noncarboxylic oxygens, primary amino groups, and finally other nitrogens. The protein backbone and the side chains of nonaliphatic residues, except for functional groups, are shown in white. Note that the direction of the helixes is not readily apparent on the surface, in contrast to the backbone drawing in Panel A. [Modified from (31) with permission.]

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require the native conformation to bring them into mutual proximity (Figure 1A,B).

In addition to the majority of antibodies that are specific for the native conformation, there is some evidence for a smaller subpopulation of antibodies with a substantially higher affinity for peptide fragments than for the native protein (12). This previously unrecognized population can be enriched by affinity chromatography on peptide-Sepharose. In a competitive radioimmunoassay with radiolabeled peptide as tracer, these antibodies require 10^5-fold more *native* myoglobin than unlabeled peptide to compete. This population may represent antibodies specific for novel sequential determinants or for the denatured or cleaved protein.

2. A new development in our understanding of the antigenic structure of myoglobin is the realization that almost any part of the surface may be antigenic. (*a*) The studies of Hurrell et al (36) and East et al (28), together with the evidence cited above (29) that 30–40% of antibodies raised in goat, sheep, or rabbit do not bind to any of the three CNBr fragments of sperm whale myoglobin, imply that a substantial proportion of the antibodies in a polyclonal serum are directed to sites that are not defined simply by sequences I–V described earlier (21). Results of pairwise cross-reactivity studies of 13 cetacean myoglobins of known amino acid sequence and rabbit antisera to 12 of them also implied that most of the immunological reactivity was due to regions outside sequences I–V (E. M. Prager, A. C. Wilson, unpublished). (*b*) Of the 12 residues implicated in the binding sites of five monoclonal antibodies to sperm whale or human myoglobin (31,33), only two (residues 113 and 145) are within the original five sites. Most of the residues are not considered close enough in a nearest-neighbor analysis (37) to perturb these five sites; and their effects, therefore, cannot be interpreted as secondary influences on sites I–V. (*c*) A synthetic peptide corresponding to residues 72–88 of beef myoglobin (Figure 1A), containing none of the residues in sites I–V but including four residues, 74, 79, 83, and 87, implicated in the binding of monoclonal antibodies to human or to sperm whale myoglobin (31,33), bound 16% of the antibodies in a rabbit antiserum to beef myoglobin, consistent with the surface area of beef myoglobin it occupies (34). Certain monoclonal antibodies to beef myoglobin also bind to this peptide (D. Dorow, K. Haynes, P-t. Shi, P. E. E. Todd, S. J. Leach, manuscript in preparation), presumably because (unlike the five small peptides referred to earlier) it corresponds structurally to a substantial surface area in the intact myoglobin. Interestingly in retrospect, two of the chymotryptic peptides that bound to one of Crumpton & Wilkinson's (20) rabbit antisera to sperm whale myoglobin were the fragments 70–76 and 77–89. Therefore, it is clear that this area may represent another substantial antigenic region of myoglobins. (*d*) Other sites outside this area and outside the five earlier sites have been identified; for example, the synthetic peptide

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25–55 of beef myoglobin reacts with polyclonal as well as with two out of eight monoclonal antibodies to beef myoglobin (D. Dorow et al, cited above). Together, these peptides and the other sites implicated above cover a large part of the surface of the molecule.

3. Another new development in defining the antigenic structure of myoglobin is the finding that the five original sites (21) are not always recognized. In one study (38) high-titer antisera to sperm whale myoglobin raised in goats, sheep, and several strains of high-responder mice were tested for their ability to bind to synthetic peptides corresponding to residues 56–63 (site II) and 93–102 (site III). No binding to either peptide could be demonstrated either by competitive or direct binding assays over a wide concentration range. Thus, these sites are not recognized by all antisera. In fact, a general problem with the use of small peptides to define antibody specificity has come to light in a recent study (P-t. Shi, J. Riehm, P. E. E. Todd, S. J. Leach, manuscript submitted) using short synthetic peptides attached to solid-phase resins and measuring binding of ^{125}I-labeled antibodies. Athough peptides corresponding to previously reported sequential sites I–V (21) of beef myoglobin bound antibodies raised to beef myoglobin, they also bound antibodies to staphylococcal nuclease nearly as well. Moreover, the antibodies to myoglobin also bound to short unrelated peptides in a manner that implicated lysine and aromatic residues as main structures determining binding. It was concluded that the observed binding of antibodies to short and unstructured peptides of two to seven residues is of limited biological significance. Biological specificity can be assessed only with peptides that represent larger areas of the protein antigen surface and that have demonstrable conformational preferences.

4. A significant observation, which necessitates revision of the earlier postulate that the same sites are recognized regardless of the species immunized (24,25), is that antibodies to beef myoglobin raised in sheep have a fine specificity very different from that of antibodies to beef myoglobins raised in rabbits, dogs, and chickens (H. M. Cooper, I. J. East, P. E. E. Todd, S. J. Leach, manuscript submitted). This difference in response is explained by the fact that sheep and beef myoglobin differ by only six residues, whereas beef myoglobin differs substantially from the myoglobins of the other three responding species. The interpretation of such data, in common with those for cytochrome *c* responses (16), is that immunogenicity depends significantly on the difference between the immunogen and the responder's homologous protein, and is not an inherent property of the antigen alone.

5. T-cell and antibody responses to myoglobin have quite different fine specificities. For instance, antisera raised to sperm whale myoglobin in several strains of mice cross-react extensively with horse myoglobin and vice versa (39). In contrast, T cells immune to sperm whale myoglobin in three strains (B10.S, B10.D2, B10.GD) do not cross-react with horse myoglobin, and T

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cells immune to horse myoglobin in at least one strain (B10.S), which is a high responder to both myoglobins, do not cross-react with sperm whale myoglobin (40,40a). Moreover, an immunodominant site recognized by T lymphocytes from these three strains was identified, centering on residue 109. The small difference between Glu 109 of sperm whale myoglobin and Asp 109 of horse myoglobin appears to be critical for T-cell recognition (40). This specificity has been confirmed with monoclonal populations of myoglobin-specific T cells from B10.D2 mice grown in long-term tissue culture (40a). So far, no laboratory has identified any antibodies that bind to this site. Thus, sites immunogenic for T-lymphocyte responses are not necessarily the same as those immunogenic for antibody production. At the very least, the frequency distribution is skewed quite differently for the two types of responses, and the number of sites seen by T cells may be far more limited than the number recognized by antibodies.

A second, minor site around Lys 140 has been identified that is recognized by several clones of T cells from B10.D2 mice (I. J. Berkower, H. Kawamura, L. A. Matis, F. R. N. Gurd, J. A. Berzofsky, manuscript in preparation). This site does coincide with the site recognized by one monoclonal antibody (31). Interestingly, the MHC of antigen-presenting cells (such as macrophages or dendritic cells) determines which antigenic site is stimulatory for T cells. All T-cell clones studied that recognized myoglobin in association with the *I–A*-subregion-encoded Ia antigen of antigen-presenting cells were specific for the Glu 109 site, whereas all of those restricted to the *I–E*-encoded Ia molecule were specific for the Lys 140 site. Thus, the major histocompatibility antigens play a major role in this skewing or limitation of the T-cell repertoire.

LYSOZYME *c*

Chicken lysozyme *c* from hen egg-white (HEL) has long served as a prototype protein for investigating the specificity of immune recognition. HEL is a small globular protein (129 residues), unusually stable in solution, whose mode of enzymatic action has been extensively studied (41) (Figure 2). In addition, its three-dimensional crystalline structure has been determined to a high degree of precision (48). The presence of four disulfide bonds makes it likely that the structure of HEL in solution is close to that in the crystalline state. X-ray analysis of human lysozyme, which differs from HEL at 52 amino acid residues, shows that the peptide backbone is highly conserved evolutionarily (49). Thus, it is reasonable to assume that the many sequenced bird lysozymes, which are more closely related to HEL, have very similar three-dimensional structures, as has already been demonstrated for turkey lysozyme (50). However, local changes due to radical amino acid substitutions as well as subtle long-range effects are not excluded by present analyses.

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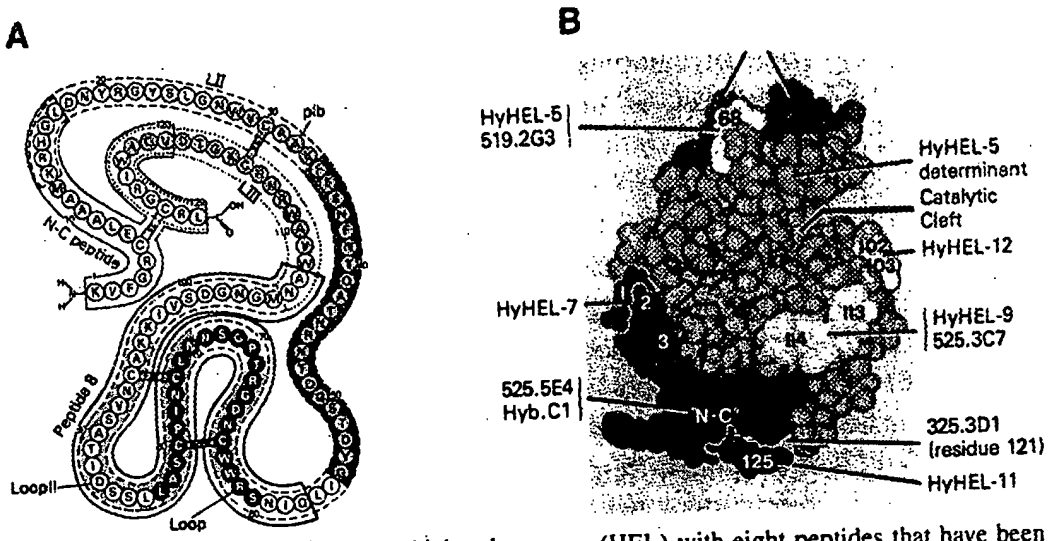

Figure 2 A: Primary structure of chicken lysozyme (HEL) with eight peptides that have been shown to be antigenic when tested against anti-HEL [reviewed in (42)]: N–C peptide, solid outline; LII, dashed outline; plb, stippled; a continuous region (amino acids 34–54) within plb, black box; peptide 8, heavy black outline; the Loop, black with white lettering; Loop II, stippled box; and LIII, dotted outline.

B: Space-filling model of HEL, computer generated as described previously (35,43). The Loop and N–C peptide are dark grey, with residues 1–3 black. Specific residues recognized by monoclonal antibodies are colored or outlined in white; a hypothesized unit determinant for antibody HyHEL–5 is outlined in a dotted black line. HyHEL antibodies are from the Smith-Gill laboratory (43; S. J. Smith-Gill, T. B. Lavoie, C. R. Mainhart, manuscript submitted). Antibody Hyb.C1 is from Fujio's laboratory (44). All other antibodies are from the Sercarz laboratory (45–47).

Several important issues have been addressed by the study of anti–HEL antibodies. One set of questions focuses on the definition of antigenic structure of the native HEL molecule. Since such studies necessarily utilize the host immune response as an assay or measure of antigenicity, it has therefore been necessary to consider a second set of questions, focusing on the nature of the repertoire of antibodies in the responding host and on the regulatory mechanisms that limit the total expressed repertoire to a skewed set of specificities that become dominant in the course of the immune response.

That the antigenicity of HEL is dependent upon intact conformational structure is supported by the observation of little or no cross-reactivity between native and denatured lysozymes (51). Purified peptides such as the N–C terminal peptide (see Figure 2A) can react with both polyclonal and monoclonal antibodies to HEL but with a far lower affinity than does HEL itself (42 and references therein; 44,51–53). The majority of antibodies do not react with any nondisulfide peptides. These studies support the conclusion that most antigenic determinants on HEL are *assembled topographic determinants*.

An antigenic region known as the "Loop," which includes residues 64–80 (see Figure 2A), has been studied extensively by the use of purified disulfide peptides. Whole goat, rabbit, and mouse antisera to native lysozymes (anti-

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lysozyme), as well as anti-lysozyme fractionated into antibodies reactive with only Loop (anti-Loop) or with all regions except the Loop, were tested against evolutionary variants of lysozyme as well as against Loop peptides. Several important conclusions concerning antigenicity of HEL have emerged from these studies (42 and references therein; 54): (*a*) The Loop participates in the antigenic structure of lysozyme, and position 68 has a major role in its specificity; (*b*) the ability of evolutionarily related lysozymes to compete with Loop for binding to anti-lysozyme or anti-Loop correlates highly with sequence changes in the Loop, while the ability to compete with native lysozymes does not; (*c*) not all residues contribute equally to antigenicity; (*d*) antigenicity of the Loop depends upon intact conformational structure which is maintained by a disulfide bond between residues 64 and 80; (*e*) the region-specific anti-Loop response in mice is under genetic control and does not necessarily correspond to the total anti-lysozyme response (55).

The nature of individual determinants recognized by monoclonal antibodies has recently been studied (43,44,45,47). One such monoclonal, described in detail by Smith-Gill et al (43), is sensitive to substitution of Arg 68 by Lys and is insensitive to a variety of amino acid substitutions in other parts of the lysozyme molecule. The authors postulate that a determinant that includes Arg 45, Arg 68, Thr 47, and Asp 48 is being recognized. This determinant is distinct from but overlaps the Loop, and is part of an anti-parallel β-sheet that was implicated previously as an antigenic region using polyclonal antibodies (53).

The question of how much of the surface of HEL is antigenic has been controversial. Most of the HEL surface was implicated in its antigenicity by an extensive study of Wilson, Prager, and colleagues (e.g. 54,56–59), which showed that the cross-reactivity of rabbit antisera to lysozymes correlated with the number of amino acid sequence differences among 15 evolutionarily related bird lysozymes tested. Another study (60) reported that in large antibody excess, five molecules of rabbit anti–HEL bound to a single HEL molecule. Based on size considerations, a maximum of six immunoglobulin molecules could theoretically bind HEL simultaneously (61). These results suggest that all faces of the HEL surface contain at least one antigenic site.

Atassi and his colleagues (62,63) used "surface-simulated peptides" to mimic surface determinants on the molecule. Three peptides were able to interact at low affinity with goat antibodies to native HEL and could absorb entirely the reactivity of these antisera. Thus, they suggested that "the precise and entire antigenic structure of native lysozyme" had been solved for all hosts. They proposed that only three antigenic sites (I—residues 5, 7, 13, 14, 125; II—33, 34, 113, 114, 116; III—62, 87, 89, 93, 96, 97) exist in HEL and, further, that all these sites are "discontinuous"—i.e. *assembled*. Their postulate of three sites contrasts with the numerous studies discussed above suggesting that

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a much larger proportion of the surface may be involved in antigenicity. Notably their sites do not include Arg 68, the Loop, and several segmental antigenic regions previously demonstrated (Figure 2A).

This view of limited antigenicity disagrees with that of a more encompassing "multideterminant" structure (4,58,59,64) suggested by previous studies discussed above. Recent reexaminations of the antigenic structure of HEL as defined by the total number of different specificities that can be detected in panels of monoclonal antibodies from several strains of inbred mice (43–45,47,47a) also suggest that the Atassi structure is incomplete. Four lines of evidence from these studies support the conclusion that the capacity for antibody response to HEL is very broad, consisting of many different clonotypes that bind distinct, but often overlapping, determinants including in aggregate most, if not all, of the HEL surface. (*a*) Studies of reactivity with panels of evolutionary variants of lysozyme, chemically modified lysozyme, and peptides have allowed the identification of specific residues and regions as critical to the binding of specific monoclonal antibodies. Antibodies specific for determinants containing the following residues have been identified: 1, 68, 121, 125, 113–114, 19–21, and 102–103. Additionally, monoclonal antibodies have been identified that bind to the N–C region, LII, or LIII (43,44,47,47a) (Figure 2B). (*b*) Most monoclonal antibodies to HEL have individually distinct patterns of fine specificity (47,47a). For example, when each of 44 monoclonal antibodies from A-strain mice were tested for reactivity with a panel of 10 different lysozymes, they could be divided into at least 18 fine-specificity groups, and only 3 pairs of antibodies with very similar profiles were found. (*c*) Most monoclonal antibodies had distinct profiles of competitive binding to HEL, and the complementation groups derived from these profiles revealed complex patterns of overlap (45,47a). These results also suggest that many individual subsites or determinants are recognized by specific antibodies binding within large regions that themselves overlap and include most of the HEL surface. (*d*) Studies on the isoelectric focusing patterns, heavy-chain variable-region gene rearrangements, and N-terminal amino acid sequences of the heavy and light chains of the antibodies themselves indicate that the antibodies express a great diversity of heavy- and light-chain variable-region genes (45,47a); such structural diversity is consistent with a broad pattern of determinant recognition.

Though the repertoire of immune responses to HEL, as represented by monoclonal antibodies, is very diverse, the actual repertoire expressed in the serum represents a skewed distribution of the total potential repertoire, such that some specificities are rare and others predominate. For example, one A-strain mouse monoclonal antibody (1G11) competed for binding to HEL with all other A-strain monoclonal antibodies tested, as well as with polyclonal antisera. The other monoclonal antibodies each competed with some, but not

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all, of the remaining antibodies, indicating that although many unique determinants are recognized, they are confined to a single face of HEL (45). However, that the apparently predominant specificities may differ among strains is indicated by complementation studies using a BALB/c hybridoma, HyHEL–5; while HyHEL–5 overlaps with most of the A-strain monoclonals tested, including 1G11 (45), it shows little overlap with most of the other BALB/c hybridomas (47a).

Further, the specificity repertoire distribution of the monoclonal antibodies differs from that found in individual antisera during both primary and secondary antibody responses (47). Thus, the "hybridoma" repertoire taken as a whole, although itself selected, may reveal a broader spectrum of the available repertoire than do serum antibodies from a given individual or inbred strain at the height of the in vivo response. For example, recent studies employing $H-2^a$ mice indicated that at least 75% of secondary response antibodies to HEL could be adsorbed on immobilized N–C peptide (52). Most such antibodies (90–95%) reacted equally well with HEL and with lysozyme lacking its three amino-terminal residues (AP–HEL) (46). In contrast, half the early primary response antibody does not recognize AP–HEL (46). These results suggest an ordered progression in recognition of different determinants on HEL, probably determined by regulatory mechanisms.

Several interesting observations have been made in studies of the role of T cells in the immune response to HEL. (*a*) While most antibodies to HEl do not react with nondisulfide peptides, segmental peptides suffice to trigger T-cell proliferative activity in all cases explored (52,61). (*b*) Mice of each MHC haplotype recognize a characteristic determinant or a small set of determinants on lysozyme. For example, $H-2^b$ helper cells seem to select a single site within the tryptic peptide containing amino acids 74–96, $H-2^d$ T cells strongly prefer a site including amino acids 113 and 114, while $H-2^a$ T cells recognize three sites but in a clearly hierarchical way (65). (*c*) The restriction of the T-helper cell repertoire can influence the population of B cells selected for expression in the antibody response (61,65). (*d*) Not only the antigen-specific but also the idiotype-recognizing T cells influence the specificity of the emerging B-cell population (66). (*e*) In addition to being highly restricted, the repertoires of suppressor and helper T cells inducible by peptide fragments seem to be nonoverlapping in the case of lysozyme (52,67). Suppressor T-cell-inducing reactivity in $H-2^b$ mice is circumscribed to a single area on the HEL molecule, at the amino terminus (46,67).

In summary, studies designed to examine total antibody repertoire, such that rare as well as predominant specificities are detected (as in cases of large antibody excess or with large numbers of monoclonal antibodies), are consistent with the hypothesis that most, if not all, of the HEL surface is potentially antigenic, consisting of multiple, overlapping determinants that may be rec-

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ognized in some hosts. In contrast, studies designed to sample the predominantly expressed specificities in individual hosts (or inbred strains) have led to the conclusion that the immune response for any given individual is skewed such that antibodies recognizing a limited number of antigenic regions on the surface of HEL will predominate. The latter result depends upon the species and genetic make-up of the host, and reflects the interplay of regulatory circuits operating in that individual's immune system.

CYTOCHROME *c*

Cytochrome *c* is a small heme protein consisting of a single polypeptide chain of 103 to 111 residues, long found to be a poor immunogen (14,15). It was only when glutaraldehyde-polymerized forms were employed that consistent and relatively strong antibody responses were obtained, making it possible to utilize the proteins of many different species to study in detail the specificities of both antibodies and T cells raised against any one of them.

Mitochondrial cytochomes *c* present a particularly attractive model system for the immunological study of globular proteins (14,15). The protein is easy to prepare, it is present in all eukaryotes, over 100 of them have had their amino acid sequences determined (68–70), and X-ray crystallographic studies have shown that all maintain the same polypeptide backbone spatial structure, the so-called *cytochrome c fold* (68,71). Differences in amino acid sequence among mitochondrial cytochromes *c* are, in effect, reflected only in local differences in surface topography resulting from the variation in side chains. This argument is particularly strong for cytochromes *c* that differ by less than 10% in amino acid sequence, with the variant residues having side chains at the surface of the protein, an ideal situation for the study of topographic antigenic determinants.

The first isolation in pure form of an antibody population directed toward a single site on cytochrome *c* was performed with rabbit antisera directed against human cytochrome *c* (72). Rhesus monkey cytochrome *c* differs from the human protein only at threonine 58, an external residue on the "back" surface of the molecule. Adsorption of anti-human antisera with the monkey protein left a population of antibodies that bound only to those cytochromes *c* with an isoleucine 58, such as the human or kangaroo proteins. Less conclusive, indirect evidence indicated that there may be three other antigenic determinants on human cytochrome *c* with respect to the rabbit host (14,15,73), in conformity with the stoichiometry of four specific Fab′ fragments binding simultaneously to the human protein, as determined by fluorescence quenching titrations (74).

Urbanski & Margoliash (16), using sequential adsorption of antisera on insolubilized cytochromes *c*, isolated three single-site antibody populations

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from rabbit antisera raised against either mouse or guanaco cytochromes *c*, as well as from ascitic fluid of mice immunized against guanaco or rabbit cytochromes *c*. The three variable positions to consider were 44, 62, and 89, at which the rabbit protein carries valine, aspartic acid, and aspartic acid; the mouse protein, alanine, aspartic acid, and glycine; and the guanaco protein, valine, glutamic acid, and glycine. Thus, in every case the immunogen differs from the host cytochrome *c* at only two residues, and in every case, antibodies specific for both of the corresponding regions were isolated. Remarkably, a third antibody population that bound to the area of aspartic acid 62, a residue shared by the immunogen and the host proteins, was present in both the rabbit antiserum to mouse cytochrome *c* and the mouse antiserum (ascitic fluid) to the rabbit protein. Farr assays showed that the guanaco protein, with a glutamic acid at 62, interacted more strongly with these antibodies than did the mouse and rabbit proteins, which carry aspartic acid at that position. This observation served to locate the binding domain of the third antibody population. The mechanism for the production of anti-self antibodies in this case, and the role that regulatory influences may play in their elicitation, are unknown. However, a simple speculation (16) was that the clones responsible for the antibodies were the products of B cells directed against the glutamic acid 62–containing surface and had not been eliminated during ontogeny because of their weak reactivity with the self-protein, with an aspartic acid 62, yet could be activated by the large doses of the cross-reacting cytochrome *c* used as immunogen.

Jemmerson & Margoliash (13) fractionated completely the rabbit antibody response to horse cytochrome *c*, isolated seven subpopulations of antibodies directed against three sites on the molecule, and showed that these comprised the totality of the horse cytochrome *c*–specific antibodies (Figure 3). Four of the populations bound a complex determinant affected by the sequence variations at residues 89 and 92, two populations bound the region of residue 44, and one that of residue 60. Competitive binding assays with a series of cytochromes *c* of varying similarity to the horse protein revealed that residues in the vicinity of the immunodominant side chains were also involved in antibody binding. These results correlate well with studies of rabbit anti-horse cytochrome *c* antisera by Berman & Harbury (76) and by Eng & Reichlin (77). The latter authors employed affinity adsorption techniques and observed the same three immunogenic areas on the protein.

The rabbit antibody response to pigeon cytochrome *c* was analyzed by Hannum & Margoliash, employing competitive plate-binding assays (C. H. Hannum, E. Margoliash, unpublished). Antibodies were found to be directed against four sites on the pigeon molecule, representing all seven of the sequence variations with rabbit cytochrome *c*, confirming fluorescence quenching stoichiometry calculations.

The first observation of a T-cell response to a cytochrome *c* was reported

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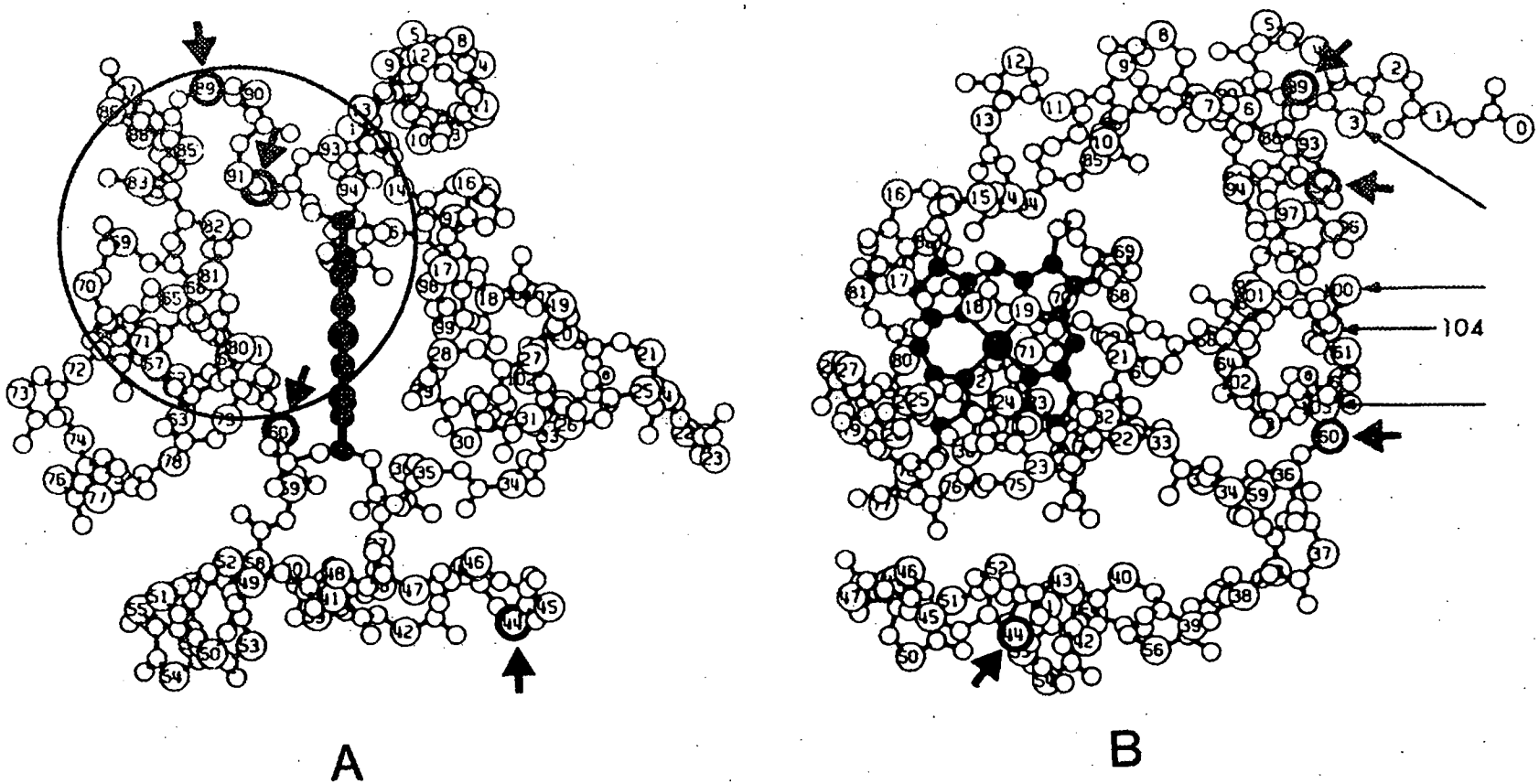

Figure 3 Diagrams of the backbone conformations of cytochrome *c*. *Panel A* is a view from the "front," with the solvent-accessible edge of the heme prosthetic group in darkened atoms. The large circle is the enzymic interaction domain. *Panel B* is a view from the righthand side, in which the square structure of the heme (darkened atoms) is seen in full. In both panels, the larger numbered circles are the α carbons. The α carbons of the residues that were shown to be immunodominant for rabbit anti-horse cytochrome *c* antibodies are indicated by heavy circles and heavy arrows. Three of the four immunodominant residues that constitute the major mouse anti-pigeon cytochrome *c* determinant (residues 3, 100, and 103) are indicated by thin arrows. The fourth, residue 104, is not shown, as the X-ray crystallographic conformation employed is taken from an electron density map of tuna cytochrome *c* (75), which has only 103 residues. However, the thin arrow marked 104 points to where that residue side chain would be located, indicating that all four immunodominant side chains are in fact in a cluster on the surface of the protein.

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by Reichlin & Turk (78), who induced delayed hypersensitivity in guinea pigs. The responses displayed the typical pattern of cross-reactivities to a variety of cytochromes *c* similar to that observed with rabbit antibodies. Fragments of the immunogen were ineffective. Contrary to the antibody response, however, guinea pigs immunized with a cytochrome *c* polymer displayed stronger hypersensitivity to polymers than to monomers, while the opposite was observed for animals primed with a monomer. Thus, T cells, unlike B-cell products (79,80), apparently differentiate between the single antigenic determinants of the monomeric protein and the clustered determinants of a polymer. Subsequent work by Wolff & Reichlin (81) suggested that the specificity of the guinea pig T cells was at least as sensitive to the amino acid substitutions on cross-reacting antigens as were rabbit antibodies. Similarly, Corradin & Chiller (82) found that mouse T cells primed with either beef or horse cytochrome *c* proliferated in response to either the native or fragmented forms of the immunogen, and that single residue substitutions could be recognized. One such site occurred at residue 89.

A comprehensive examination of both B- and T-cell responses to the same cytochrome *c* was carried out with B10.A mice immunized with the monomeric pigeon protein. Using a proliferative T-cell assay, Solinger et al (83) mapped the genes governing responsiveness to monomeric pigeon cytochrome *c* to both the *I–A* and *I–E* subregions of the mouse major histocompatibility complex. Subsequent experiments with monoclonal antibodies have shown that such a response indeed requires the Ia molecule $I\text{–}A^{k}_{e\beta}$:$I\text{–}E^{k}_{\alpha}$ or the hybrid Ia molecule $I\text{–}A^{s}_{e\beta}$:$I\text{–}E^{k}_{\alpha}$ (84,85). Cross-reactivity experiments with a large variety of cytochromes *c* and their cyanogen bromide fragments showed that the region of the molecule responsible for T-cell activation contains the glutamine at position 100 and the carboxyl-terminal lysine at position 104, with the possible added involvement of isoleucine 3 (86). An analysis of the B-cell response of the same mice to pigeon cytochrome *c* showed that the majority of the antibodies bound to the region of residues 3, 100, 103, and 104, a complex determinant that, although not identical to the major T-cell determinant, completely overlaps with it (C. H. Hannum, L. A. Matis, R. H. Schwartz, E. Margoliash, unpublished). These residues are all located within a few angstroms of one another and form an assembled topographic determinant containing segments of the carboxyl-terminal and amino-terminal α-helixes (Figure 3). It was also clearly demonstrated that all the antibodies are directed against those regions of the molecule where pigeon cytochrome *c* differs from the mouse protein. A comparison of the concentrations of pigeon and mouse cytochromes *c* that produce 50% inhibition of binding allows the calculation of the contribution of the variant side chains to the binding energy of the antibody-antigen interaction, amounting to about −3 kcal/mole (87). If the average affinity constant, K_a, for pigeon cytochrome *c* is about 10^8 M^{-1},

the protein.

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then the −3 kcal/mole would constitute some 30% of the total energy of interaction. The remaining energy is apparently provided by the interaction with the surface of the protein surrounding the immunodominant residues, areas identical on both immunogen and host proteins. This is probably the basis for the extensive cross-reactivities always observed when antibodies raised against a particular cytochrome *c* are tested with heterologous cytochromes *c*, including the host protein.

It should be noted that whereas antibodies raised in rabbits and mice to native horse or pigeon cytochromes *c* showed much lower affinities for peptide fragments of the immunogens (13,87), T cells from mice primed with horse, beef, or pigeon cytochrome *c* responded as well, and sometimes better, to the fragmented forms in proliferation assays (82,83,86). This is in contrast to the failure of guinea pigs to display delayed hypersensitivity following challenge with fragments of the horse protein (78). The differences between B- and T-cell reactivities with immunogen fragments could reflect a smaller T-cell receptor interaction domain, which makes it possible for peptide segments to react as well as they do in the native immunogen.

The one result that varies from all others was reported by Atassi (88), who found that antibodies raised in two rabbits against beef cytochrome *c* all bound to an insolubilized synthetic peptide comprising residues 42–50 of beef cytochrome *c*, a segment spanning one of the four variant residues between the beef and rabbit proteins, namely proline 44. Two other insolubilized synthetic peptides covering the other three variant residues did not bind any of the antibodies. It is not obvious why antibodies directed against the other potential determinants on beef cytochrome *c* were either not generated or not detected in these experiments, when, as discussed above, in every other case examined, rabbits produced antibodies to every one of the areas of the protein containing residues that differed from those in rabbit cytochrome *c*.

That cytochromes *c* identical to that of the homologous host protein are also capable of eliciting an antibody response became evident when rabbits injected with acetylated γ-globulin-conjugated (89) or glutaraldehyde-polymerized rabbit cytochrome *c* (90) yielded rabbit cytochrome *c*–specific antibodies in amounts averaging about 10% of the response to heterologous cytochromes *c*. Fractionation by affinity adsorption, and analysis for specificity by competitive binding assays (90), showed that these antibodies are not directed against all or many sites on the self immunogen, but rather against three areas that contain the segments of the protein that have varied most recently in the course of the evolutionary descent of mammalian cytochromes *c*. Jemmerson & Margoliash have speculated (90) that such remarkable antibody specificities represent a breakdown of self-tolerance resulting from the stimulation of cross-reactive clones, the repertoire for which has not yet been eliminated by the evolutionary selective process that may govern the retention of such autoimmune poten-

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tialities. Whether this or some other process is involved in these phenomena has not yet been examined.

In summary, cytochrome *c* can elicit antibodies in rabbits and appropriate strains of mice that react to every one of the areas in which the immunizing cytochrome *c* has one or more side-chain differences from the cytochrome *c* of the host. Furthermore, antigenic determinants responsible for T-cell proliferation appear to have the same general specificities. However, in some cases antibodies have also been obtained, albeit with great difficulty, to areas of the immunizing protein identical to those of the host protein. The mechanisms underlying such self-immunity phenomena have not been examined.

SERUM ALBUMIN

Serum albumin (Figure 4) is a single polypeptide chain of approximately 582 amino acids. Its three structural and functional domains are phylogenetically related (91) and are independent folding units (93). Although homology in general structure exists among domains, the amino acid sequence and function of each domain have diverged (91). For example, there is at most 25% sequence identity between any two domains and no more than 10% among all three. Most of this identity among all three domains (about $\frac{12}{19}$ conserved residues) centers around the disulfide bonds, which are highly conserved throughout evolution (91). Indeed, $\frac{18}{19}$ residues shared among the domains of bovine serum albumin (BSA) are shared between it and human serum albumin (HSA). Serum albumin has long been a standard tool for the immunologist, the immunochemist, the protein biochemist, and the evolutionary biologist, who uses albumin antisera to estimate the genetic relatedness and times of divergence of species. Yet until recently, little has been known about its chemical and physical structure, not to mention its detailed antigenic structure or the control of immune responses to this protein.

There seems little doubt that serum albumin is a multideterminant antigen for which each determinant is unique, i.e. each occurs only once in the albumin molecule. This concept of multiple, distinct, antigenic determinants on serum albumin is supported by a vast literature spanning three decades beginning with the pioneer work on human serum albumin by Lapresle (94). In these early studies, Lapresle demonstrated that various enzymes would degrade HSA into several antigenically distinct fragments. Porter and his group (95,96) have shown that fragments from BSA and HSA bear only a portion of the total antigenic determinants present on the intact molecule. Using a variety of techniques Weigle (97) and others (98–100) have shown many distinct antigenic determinants on BSA. Other studies on antibody synthesis by single cells (101), on acquired immunological tolerance to albumin (102), and on

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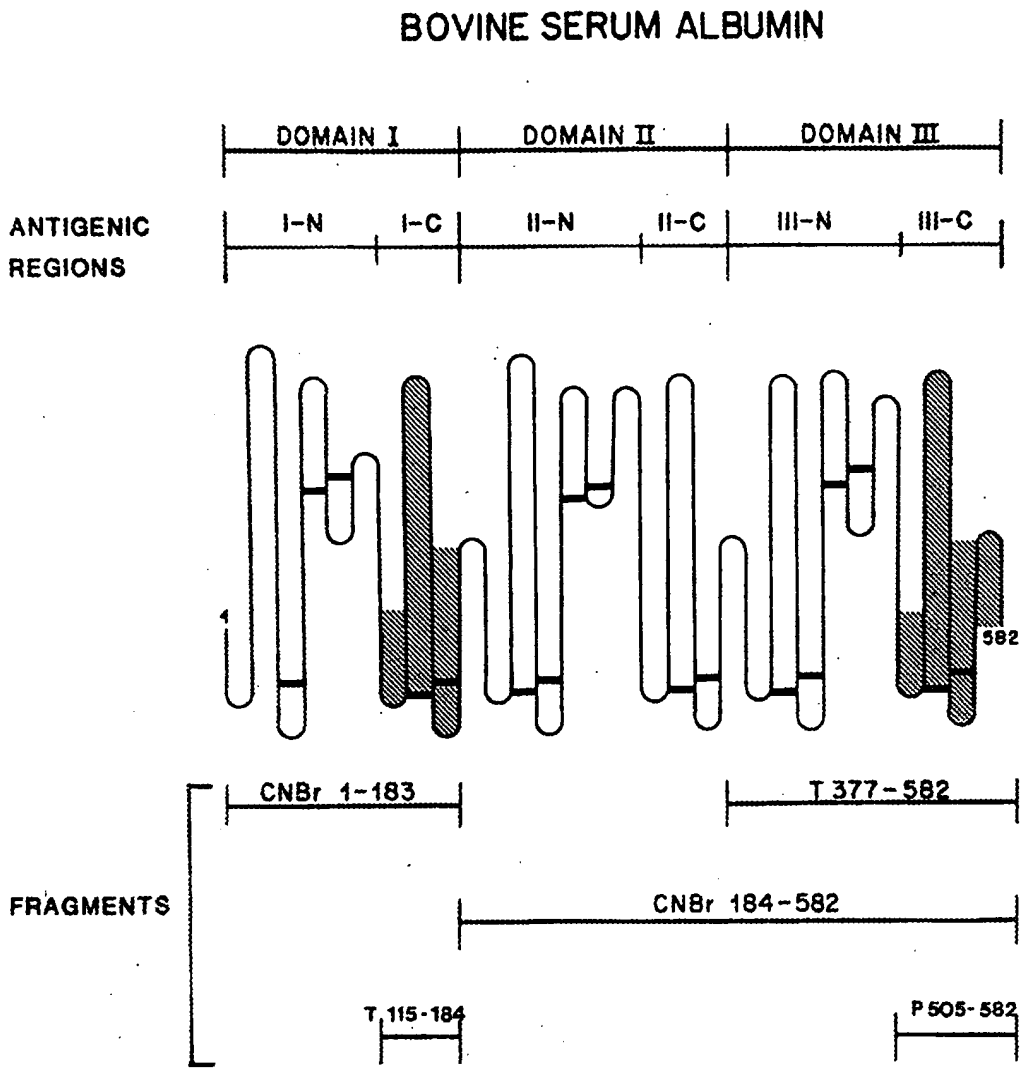

Figure 4 Diagrammatic representation of the structure of bovine serum albumin as adapted from Brown (91). Antigenic regions and antigenically active fragments are as described by Benjamin & Teale (92) and references therein.

control of immune responses to BSA by restricted populations of T cells (103), support this multi-distinct-determinant nature of albumin.

A more recent series of studies on BSA (92,104 and references therein; 105,106) and HSA (107) has yielded essentially identical results. Using polyclonal antisera to intact albumin and antigenically active fragments of albumin, each laboratory has demonstrated albumin to be composed of several antigenic regions (see Figure 4), each of which has one or more distinct determinants. Some evidence of cross-reactivity among domains, involving a small proportion of the antibodies, has occasionally been found (105,107). Similar results have been obtained with T-cell responses to BSA and fragments representing the domains and subdomains of the molecule (108). Thus, the

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determinants on serum albumin are multiple and distinct at both the B-cell and the T-cell level.

Also recently, a series of studies was initiated using monoclonal antibodies and T-cell clones (104,109; L. D. Wilson, R. L. Riley, D. C. Benjamin, manuscript submitted; D. C. Benjamin, K. DeCourcy, manuscript submitted). Two hundred hybridoma cell lines producing antibody to BSA were established, and 64 randomly chosen clones have been fully characterized. In no instance did a monoclonal antibody react with more than one domain or subdomain. The 64 monoclonal antibodies could be placed into 25 groups based on their cross-reactivity with a panel of 10 mammalian albumins, suggesting a minimum of 25 different determinants. If, in addition to cross-reactivity patterns, the domain or subdomain specificity of each monoclonal antibody was considered, then a minimum of 33 determinants could be demonstrated. In other words, two or more monoclonal antibodies may have the same cross-reactivity pattern but be specific for distinct regions of the BSA molecule and thus define two, or more, distinct determinants rather than one as defined by cross-reactivity alone. Thirteen of the 64 monoclonal antibodies define 13 nonoverlapping antigenic determinants as determined by competitive inhibition assays. The remaining 51 monoclonal antibodies competitively inhibit the binding of one or more of these 13 monoclonals in a manner consistent with the existence of multiple overlapping antigenic determinants. These studies have been partially confirmed in at least one other laboratory (110). In addition, long-term T-cell lines specific for BSA are exquisitely specific for a single subdomain (109; L. D. Wilson, R. L. Riley, D. C. Benjamin, manuscript submitted). One might argue that during production of monoclonal antibodies or T-cell clones those specific for minor determinants were preferentially isolated. However, the fact that not a single monoclonal reagent has been found that cross-reacts among domains makes that possibility highly unlikely.

These results contrast with those described by Atassi and his colleagues (111,112), who have suggested that serum albumin contains only two determinants, that these two determinants are present on the carboxy-terminal double-disulfide loop of a domain, and that they are shared among domains, i.e. two determinants repeated three times on the entire molecule. However, these conclusions are inconsistent with other reports from their laboratory showing that: (*a*) sera taken early after immunization do detect multiple determinants (113); (*b*) although the reaction of late antisera (day 398 after immunization) with BSA is inhibited almost entirely (80–100%) by fragments representing single domains, using immunoadsorbent techniques a maximum of 50% of the anti-BSA was reactive with any one fragment (112); and (*c*) in cross-reactivity studies (100) these same late antisera showed varying degrees of cross-reactivity with a series of heterologous albumins, demonstrating multiple (not two) distinct determinants on BSA.

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Further doubt is cast on the suggestion of only two determinants by the fact that BSA probably differs from the albumin of the responding rabbit by many residues. Rabbit and cow diverged about 80 million years ago, which is about the time cow, human, and rat diverged from one another (114). Assuming a constant rate of albumin evolution (114), the rabbit-cow difference can be estimated from the known cow, human, and rat sequences (115,116), whose average pairwise difference is 160 amino acid residues. Given the assumption discussed above for cytochrome *c* that the responding animal will produce antibodies to all areas of the antigen different from its own protein, the calculation of White et al (59) that essentially all differences between the antigen and a similar protein from another animal are immunologically detectable (see the discussion section, below), and the fact that similarities among all three albumin domains are to a great extent conserved throughout evolution, we would predict many more than two determinants.

Certain other papers have been cited as supporting the repeating determinant hypothesis (111,112). For example, Peters et al (105) have suggested six distinct antigenic regions defined by distinct determinants. They have also shown that a portion of their antibody cross-reacts with fragments representing different domains. For instance, antibody reactive with fragment P307–582 (a peptic fragment containing amino acid residues 307–582) was partially inhibited in its reaction with BSA by fragments CNBr1–183 and P1–306, suggesting that the amino-terminal and carboxy-terminal portions of BSA share determinants. In direct contradiction to this result, they found that fragment P1–385 does not inhibit the same reaction although it would be expected to bear all the determinants present on the previous two inhibitory fragments. In addition, their studies show that antibody to native BSA reactive with P1–306 is not significantly inhibited by fragments from the carboxy-terminal half of BSA.

Thus, the evidence from many laboratories, published over three decades, supports the hypothesis that serum albumin contains multiple, distinct, sometimes overlapping antigenic determinants that do not repeat from domain to domain.

Serum albumin has been used more extensively than any other protein for immunological estimation of the extent of genetic divergence among species (e.g. 114,117). Albumins from thousands of pairs of vertebrate species have been compared immunologically (chiefly by quantitative micro-complement fixation—cf 114,118), and the results have been used to establish approximate time scales for the genealogical trees by which these species are related, thereby permitting a more quantitative approach to the study of evolution than was possible before. The evidence now presented for the existence of multiple determinants on albumin provides additional, strong justification for the use of albumin antisera for quantitative evolutionary studies.

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DISCUSSION—A MULTIDETERMINANT-REGULATORY MODEL

Currently there are opposing views of what constitutes a determinant on a protein antigen. One proposal is that proteins contain a very limited number of sites that are intrinsically immunogenic, irrespective of the host responding to the antigen (21,24,25,62,63,88,111–113). A contrasting view is that most of the accessible surface of any globular protein is potentially immunogenic, that one can define which sites are immunogenic only with respect to a particular responding individual, and that the total antigenic structure of a protein is the sum of all sites recognized by a large variety of responding individuals and species. The evidence summarized above strongly supports the second view and requires that we extend what has been called the "multideterminant hypothesis" (4,58,59,64) to encompass the following concepts: (*a*) The surface of a protein is essentially a continuum of potential antigenic sites; and (*b*) the structural differences between the antigen and self-protein as well as the host's immunological regulatory mechanisms are the important factors influencing the outcome of the immune response. We term this extended hypothesis the "multideterminant-regulatory model."

Antigenic determinants are defined by the specificities of the antibodies to that antigen. Because the complexity of the antibody response varies with the degree of antigen foreignness (16), as well as type of antibody studied (i.e. polyclonal versus monoclonal), varying views of the definition of an antigenic determinant have arisen. Determinants have historically been considered to be discrete, nonoverlapping portions of the surface structure, and to consist of a definable, finite number for a given protein in a given host (119). For highly conserved proteins such as cytochrome *c*, polyclonal antisera can be fractionated into distinct, nonoverlapping specificities that correspond to sites of sequence variation. On the other hand, experiments designed to examine the full repertoire of monoclonal antibody specificities to protein antigens have yielded complex patterns of overlap, and these support the hypothesis that the surface of a protein molecule is a complex array of overlapping antigenic determinants. From the present data, one cannot exclude any surface elements from the antigenic structure of a globular protein. Therefore, any antigenic determinant can only be operationally defined as that region of the protein surface bound by a particular antibody molecule, and this may be termed the *unit antigenic determinant*.

The multideterminant-regulatory model predicts that nearly all evolutionary substitutions would directly affect immunological cross-reactivity. Table 1 summarizes experiments in which rabbits were immunized with a particular protein from one organism and the polyclonal antisera then tested for immunological cross-reactivity with evolutionary variants of that protein. A measure

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of immunological distance derived from the observed degree of cross-reactivity (56,64,114,118,120) shows a statistically highly significant linear correlation with the number of amino acid differences between each pair of proteins tested (Table 1). From the square of the coefficient one estimates that about 80% of the variation in the immunological cross-reactivity is ascribable to variation in the number of amino acid differences. A similar correlation exists for other, less extensively studied proteins (59,114). More convincing statistical data stem from experiments with small proteins differing by only one to five amino acid substitutions per 100 residues, which can routinely be distinguished with the quantitative micro-complement fixation technique (118) and polyclonal antisera. Table 2, which shows the results of an analysis of 14 independent pairs of closely related proteins, indicates that if the fraction of potentially antigenic residues were small, e.g. if F were 0.15, there would be little chance ($P < 0.0005\%$) that every pair of these proteins would be antigenically nonidentical. By contrast, if F were 0.80, the chance of every pair being nonidentical would be high ($P = 48\%$). The observation that every pair tested is nonidentical is thus consistent with F being in the vicinity of 0.80. Most amino acid substitutions are, therefore, immunologically detectable. Direct evidence for this conclusion comes from experiments with highly conserved small proteins such as cytochrome *c* (discussed in detail above); in a host whose own cytochrome *c* differed by only one or a few residues from the immunogen, every amino acid substitution could elicit antibodies.

Table 1 Dependence of immunological distance on the number of differences in amino acid sequence for 290 pairs of proteins

Protein (and reference)	Number of Pairs Tested	Correlation Coefficient[a]
Lysozyme *c* (58)[b]	106	0.95
Ribonuclease (64)	60	0.92
Myoglobin[c]	78	0.87
Cytochrome *c* (56)	23	0.87
Azurin (120)	13	0.85
Serum Albumin[d]	10	0.96

[a]Correlation coefficients were calculated from least-squares lines relating the number of positions in the amino acid sequence at which these proteins differ to the degree of immunological difference measured with polyclonal antibodies produced in rabbits. The quantitative microcomplement fixation technique (118) was used in all cases except for cytochrome *c*, done by macrocomplement fixation, and serum albumin, where the quantitative precipitin method as well as micro-complement fixation was employed.

[b]E. M. Prager, A. C. Wilson, unpublished work on additional bird, reptile, and mammal lysozymes.

[c]E. M. Prager, A. C. Wilson, unpublished work on 13 whale, dolphin, and porpoise myoglobins and antisera to 12 of them.

[d]E. M. Prager, A. C. Wilson, V. M. Sarich, unpublished calculations. The immunological comparisons are from references 97 and 121 and V. M. Sarich, unpublished measurements. The complete sequences of cow, human, and rat albumins were considered along with partial sequences (164 to 469 amino acids) of sheep, pig, horse, baboon, and mouse albumins. We thank J. R. Brown, T. Peters, Jr., and A. Dugaiczyk for personal communications and summaries concerning published and unpublished sequences.

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Table 2 Immunological detectability of amino acid substitutions in small monomeric globular proteins of known three-dimensional structure[a]

Protein	Number of Independent Pairs Tested	Detectability of Substitutions		
		Expected		Observed
		F = 0.15	F = 0.80	
Lysozyme *c*	4	2.1	77	100
Ribonuclease	4	3.2	79	100
Myoglobin	5	1.4	79	100
Azurin	1	48.0	100	100
All Proteins	14	0.00045	48	100

[a]Following the method of White et al (59), the expected values in the table indicate the probability in percent that pairs of closely related proteins (differing by 0.7–8.5% in sequence) will be immunologically distinguishable if *F*, the fraction of antigenic residues in the molecule, is 0.15 or 0.80. For each protein the product of the probabilities for the indicated number of independent pairs is given. The experimentally observed values, likewise given in percent, are the number of distinguishable pairs found relative to the number examined. The data and calculations are from references 58, 59, 64, and 120 and from footnotes *b* and *c* to Table 1; all data were obtained with the micro-complement fixation technique (118).

Some evolutionary substitutions may influence antigenicity through long-range effects, either conformational or electrostatic (122,123), as observed in allosteric proteins such as hemoglobin. Even for hemoglobin, however, less than 5% of such substitutions appear to cause long-range effects (124). For monomeric globular proteins, most evolutionary substitutions probably have only local effects on structure and function (31,33,43,124a,124b). Indeed, for several proteins discussed above, substitutions that markedly affect the binding of a monoclonal antibody to one site do not affect the binding of a second monoclonal antibody to an adjacent site (31,33,43; S. J. Smith-Gill, C. R. Mainhart, T. B. Lavoie, manuscript submitted) and vice versa. The long-range hypothesis would have predicted both sets of substitutions to perturb the binding of both antibodies, and this does not appear to occur.

The use of monoclonal antibodies has allowed the delineation of antigenic structure at a level of precision not previously possible. One can now study the specificity of each monoclonal antibody individually and thus avoid the ambiguity present in whole antisera or even antisera fractionated into populations directed to single antigenic sites. While the specificity of polyclonal antibodies for cross-reacting proteins is generally related to the number of amino acid differences or "evolutionary distance" between the two proteins (56–59,64,114,118,120; Table 1), the specificity of a monoclonal antibody is not, because a monoclonal antibody recognizes only a single site and not an average of a number of determinants summed. The sensitivity with which single amino acid substitutions can be detected is much greater with monoclonal antibodies than with antisera in which a host of other antibodies not affected by the substitution may swamp out any effects on overall binding.

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This sensitivity has allowed the delineation of boundaries of determinants (43), i.e. an amino acid substitution that affects the binding of antibody A but not B is not within the unit determinant recognized by antibody B (31,33,43). However, the precision of these boundaries is limited and dependent upon the availability of related proteins with amino acid sequence changes at each relevant border of the site. Such an analysis can be done only with difficulty using heterogeneous antisera that have been fractionated into more homogeneous populations.

Similarly, studies of competition between pairs of monoclonal antibodies for binding simultaneously to the same monomeric protein antigen often allow the grouping of antibodies into groups such that members of each group compete with one another but not with members of another group (45,125,126). This approach has allowed definition of boundaries of nonoverlapping unit determinants for myoglobin (125), lysozyme (45; S. J. Smith-Gill, C. R. Mainhart, T. B. Lavoie, manuscript submitted), and serum albumin (104,109; D. C. Benjamin, K. DeCourcy, manuscript submitted). However, these competition experiments have also frequently suggested complex patterns of overlap. For instance, numerous cases of three antibodies, e.g. called A, B, and C, interacting in a pattern such that A competes with B, B competes with C, but A does not compete with C, have been found with HEL (45; S.J. Smith-Gill, C.R. Mainhart, T. B. Lavoie, manuscript submitted), and BSA (109; D. C. Benjamin, K. DeCourcy, manuscript submitted). Such complex patterns have allowed the conclusion, on the one hand, that most of the antibodies in the secondary response to HEL by A-strain mice recognize unit antigenic determinants on a single face of the HEL surface, each antibody with its distinct pattern of fine specificity (45), and on the other hand, that most, if not all, of the BSA surface is recognized by mouse monoclonal antibodies (104,109; D. C. Benjamin, K. DeCourcy, manuscript submitted). Observation of these complex patterns of overlap is consistent with the existence of overlapping determinants and with the multideterminant-regulatory hypothesis. In three of the four protein system discussed in this review, monoclonal antibodies were found that react with overlapping determinants that in aggregate cover large portions, if not all, of the protein surface. In the case of the pigeon cytochrome *c*, a significant portion of the binding energy is due to invariant residues adjacent to the variant residue used to localize the site (87). Because a single amino acid residue may be contained in several overlapping unit determinants, the number of discrete determinants is probably greater than the number of surface residues. As a result, a number of overlapping determinants probably have not yet been distinguished from each other. These observations exclude any hypothesis that restricts the number of determinants to a few discrete sites on the surface of a protein antigen (21,62,63,88,111–113).

Important examples illustrating many of the above points are the immune

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responses to two surface glycoproteins of the influenza virus (126–129). The antigenic determinants of the neuraminidase molecule are overlapping and cluster on the distal surface of the molecule, forming a "nearly continuous surface across the top" (127). The antigenic determinants of the hemagglutinin protein similarly cluster on the distal end (126,128,129). In one study (128), 104 distinct fine specificities could be distinguished among antibodies that recognized four antigenic regions delineated by Wiley et al (126) at which antibody binding could select for viral mutations. It was estimated that the total repertoire included a minimum of 1500 fine specificities (128). In another study (129), 125 monoclonal antibodies raised to the hemagglutinin of influenza virus H3N2 cross-reacted with 15 other strains in a way that permitted mapping of 10 overlapping regions covering the entire accessible surface.

Thus, a nearly limitless specificity spectrum of antibody reactivity can potentially be elicited. However, the entire spectrum is rarely, if ever, seen in any individual. The pertinent question is what determines which of the many specifically reactive clones are expressed. Elimination or anergy of self-reactive clones would severely reduce this spectrum, especially in those instances where the immunogen is derived from a source closely related to the responding species. In the case of cytochrome *c*, a slowly evolving protein, the limited sequence differences between each cytochrome *c* and that of the responding host (2–10%) restricts the number of determinants detectable in any host. However, the fact that most substitutions that occur are immunologically detectable in some host supports the hypothesis that virtually all the surface is immunogenic. The relative simplicity of the response to closely related heterologous cytochromes *c* allowed the separation of antibody populations directed to simple or complex topographic determinants and even of several subpopulations reacting with subsites of a single complex determinant (13,16). These not only gave definitive evidence that the sequence variations between antigen and the homologous host protein were responsible for immunogenicity, but also provided the first demonstration of an overlapping distribution of determinants.

When the immune response appears to be directed predominantly toward particular antigenic regions, this may reflect limitation of the specificity repertoire by the regulatory mechanisms operative in that individual at the time of immunization. For instance, MHC-linked genes have been found to control the fine specificity of antibody and/or T-cell responses in mice and guinea pigs to a variety of protein antigens, including myoglobin (130,131), serum albumin (104,108,109,132; L. D. Wilson, R. L. Riley, D. C. Benjamin, manuscript submitted), lysozyme (133–135), cytochrome *c* (82,83), insulin (136–138), and staphylococcal nuclease (139,140). Furthermore, the predominant determinants recognized by T cells may be different from and frequently fewer than those predominant determinants recognized by antibodies in the same

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individual (40,40a,52,134). The antigenic sites utilized by antigen-specific helper T cells in a particular individual can influence the specificity of the subsequent antibody response to that antigen (52,61,67,141). Last, the expressed repertoire of an individual may be regulated by idiotypic networks (66,142).

Studies with polyclonal antibodies have suggested that protein conformation is critical to the integrity of antigenic determinants (4,5,8,27–29,51). A colorful example of the conformational specificity of polyclonal antisera is that of antibodies that distinguish the conformation of apomyoglobin from that of holomyoglobin (119). When brown holomyoglobin was reacted with antibodies to apomyoglobin, the precipitate was white. Thus, the antibody shifted the equilibrium toward a form in which the heme was excluded.

The analysis of individual determinants recognized by monoclonal antibodies has shown that a given antigenic site may include amino acids quite distant in the primary sequence that are brought close together on the surface during folding of the polypeptide chain (31,33,36,43,62). This is to be expected because an antibody sees a continuous surface, as in the space-filling models shown in Figures 1B and 2B.

The lower affinity usually observed for binding of peptide fragments of an antigen to an antibody raised against the native antigen (27,29) may be due either to the absence in the peptide of a portion of the determinant, to conformational differences between the peptide and the native molecule, or to the absence in the peptide of long-range effects (e.g. electrostatic) that may exist in the native protein (122,123). Thus, if a peptide that contains only a portion of the antigenic determinant is presented to the antibody, even if it were in the same conformation as in the native protein, the binding should be much weaker. However, in general, binding of anti-protein antibodies to short and unstructured peptides should be interpreted with caution, since recent experiments (P-t. Shi, J. Riehm, P. E. E. Todd, S. J. Leach, manuscript submitted), discussed in detail in the section on myoglobin, above, have demonstrated that such binding may be of limited biological significance, but rather dependent on hydrophobicity and charge.

In contrast to studies with antibodies, T cells elicited by immunization with a native protein frequently have been found to react equally well when challenged with either the native or denatured forms of that antigen or its peptide fragments (131,134,137,143,144). The molecular mechanism(s) of this cross-reactivity remains to be elucidated.

CONCLUSIONS

The surface of a protein antigen consists of a complex array of overlapping potential antigenic determinants; in aggregate these approach a continuum. Most determinants depend upon the conformational integrity of the native

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molecule. Those to which an individual responds are dictated by the structural differences between the antigen and the host's self-proteins and by host regulatory mechanisms, and are not necessarily an inherent property of the protein molecule reflecting restricted antigenicity or limited antigenic sites.

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SRNT

Epitopes on Protein Antigens: Misconceptions and Realities

Minireview

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Of an almost infinite variety of epitopes (or antigenic determinants) on protein molecules, only five complete structures are known. The single method that can determine the complete structure of an epitope is preparation of a complex of a monoclonal antibody Fab fragment with its antigen, crystallization of this complex, and determination of its structure using X-ray diffraction methods. This has now been done for three complexes of Fab–lysozyme (from chicken egg white) and two of Fab–neuraminidase (from influenza virus) (Amit et al., 1986; Sheriff et al., 1987; Padlan et al., 1989 and Tulip et al., 1990). These epitopes all occupy large areas comprised of 15–22 amino acid residues on several surface loops. Antigenicity of these epitopes is absolutely dependent upon conformation of the native proteins.

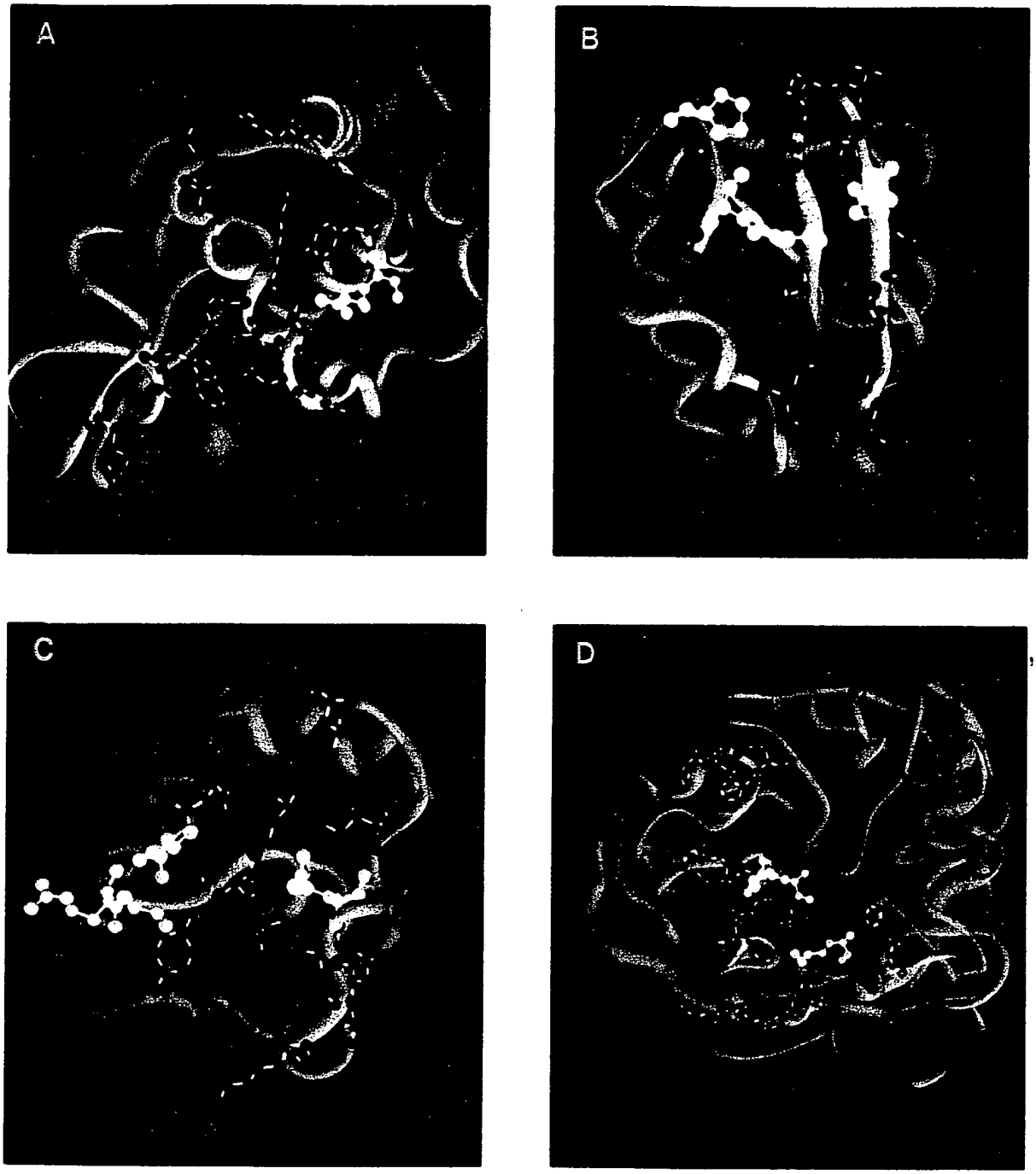
The term epitope was coined by Niels Jerne in 1960, when he proposed that "an antigen particle carries several epitopes (= surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas). Hidden epitopes which become immunologically available only after breakage, decomposition, or denaturation of the antigen are cryptotopes." Inherent in this definition is the concept that an epitope occurs on the surface of a native protein, whereas a linear peptide sequence seen after protein unfolding could occur on the inside or the outside of the folded polypeptide. Processed peptides that would be recognized by T cells in association with major histocompatibility complex molecules would actually be included in the latter category and are more appropriately considered cryptotopes rather than T cell epitopes as they are now known. Unfortunately, the term T cell epitope is in common usage to describe the peptide sequence on the original protein, although it is not in this native form that it is recognized by the T cell. In contrast, antibodies do recognize epitopes on native proteins, and the complementary paratope on the antibody is exquisitely specific for the native conformation. We propose that the term epitope be reserved for those structures on native proteins that bind antibodies, and the structures on unfolded proteins and those recognized by T cells should be termed something else.

For protein antigens, it was later proposed that epitopes might be subdivided into sequential epitopes (involving a single continuous length of the polypeptide chain) and conformational epitopes, in which several discrete amino acid sequences, widely separated in the primary structure, come together on the surface when the polypeptide chain folds to form the native protein (Sela, 1969). As discussed below, all five of the structurally defined epitopes are of the conformational type. As has been discussed previously (Benjamin et al., 1984), all determinants recognized by antibodies are conformational in that antibodies will bind with measurable affinity only to those molecules presenting the right conformation; "discontinuous" is a more accurate description of nonlinear epitopes since they are assembled from residues from several different portions of the polypeptide chain.

The five epitopes characterized by X-ray crystallography are of the discontinuous type, encompassing two to five surface loops. The highly complementary interface between antigen and antibody is absolutely dependent on the folding of the native protein. The antibody combining site is seen to be an irregular, rather flat surface with protrusions and valleys formed by amino acid side chains. The epitopes each contain between 15 and 22 residues on the antigen in contact with a similar number of residues on the antibody paratope. Although the interpretation of contacts is somewhat arbitrary (and may differ between laboratories), and the contribution of each to the binding energy is unknown, these five examples provide the best descriptions of epitopes on native proteins so far available. The figure shows the visualization of one epitope on influenza virus neuraminidase and three on lysozyme. Each epitope has a buried surface area on the antigen of 650–900 Å^2. There are 75–120 hydrogen bonds between the antibody and antigen, as well as salt links and hydrophobic interactions.

In contrast to the consensus that X-ray structures have given of protein epitopes, there still is found in the literature a widely held misconception that epitopes on native proteins consist of segments of about 6 amino acid residues that can be mimicked or mapped utilizing synthetic peptides of a similar length. In fact, numerous studies (e.g., Green et al., 1982) claimed to have localized epitopes on native proteins by studying synthetic peptides corresponding to short linear sequences within the protein. However, the success of such approaches with well-defined antibodies has been limited, and any cross-reactivity seen in these experiments probably represents binding to a proportion of denatured protein (Jemmerson and Blankenfeld, 1989). Similarly, those cases in which peptides appear to give good cross-reactivity with anti-protein antibodies in solution assays probably represent antibodies originally elicited by denatured protein.

The still-prevailing notion that a protein epitope has the size of a hexapeptide came from extrapolation of studies with carbohydrate antigens. A recent article (Goodman, 1989) describes epitopes as spanning 4–7 amino acid residues on the antigen. This concept has also received



Ribbon Diagrams Showing the Location of Epitopes

Structures were determined by X-ray diffraction analysis of crystalline complexes of monoclonal antibody Fab fragments and the antigens, hen egg white lysozyme and influenza virus neuraminidase. (A) HyHEL10–lysozyme. (B) HyHEL5–lysozyme. (C) D1.3–lysozyme. (D) NC41–neuraminidase. Residues contacting antibody light chains, heavy chains, or both are shown in red, blue, and white, respectively. Contacts for the Fab–lysozyme complexes are based on Amit et al. (1986), Sheriff et al. (1987), and Padlan et al. (1989), as summarized by Davies et al. (1990). The Fab–neuraminidase contacts are those listed by Tulip et al. (1990). The program used was Ribbons, written by Dr. M. Carson. Models were constructed by Dr. Ming Luo.

apparent support from binding studies with anti-peptide antibodies. These indicate that antibodies generated against peptides would bind to proteins in assays such as ELISA, a test in which there is good opportunity for complete or partial unfolding of the protein antigens (e.g., Green et al., 1982). Perhaps a better term for determinants detected under these conditions is unfoldon. The term epitope should be reserved for those determinants recognized by antibodies on native proteins. How can one determine if a particular antibody binds to an epitope on the native pro-

tein or to an unfoldon? For a protein to have biological activity (e.g., enzymatic activity) it needs to be correctly folded. It follows that if an antibody affects that activity, it must be binding to an epitope on the native protein, i.e., a foldon, and not to a cryptotope or unfoldon on the denatured form. Of course, many antibodies will bind to epitopes on native proteins without affecting biological activity.

On the other hand, immunization with peptides can be very useful for producing antibodies for identification of denatured or unfolded molecules (for example, on a gel or in a bacterial expression library). It must be emphasized that these antibodies are not recognizing epitopes in the sense as defined by Jerne, but simply short, linear sequences within the unfolded protein which at most could correspond to cryptotopes as defined by Jerne. Peptides are of limited use in epitope mapping or the study of antigenic structures of native proteins. In fact, in most cases anti-peptide antibodies or unfoldons identified by them are devoid of any biological significance, and results from such epitope mapping studies can be extremely misleading.

Another frequently held misconception is that proteins display only a restricted small number of epitopes in a polyclonal response. Although some specificities may be immunodominant owing to immune regulation, the evidence that most, if not all, of the protein surface is antigenic has been considered previously in detail (Benjamin et al., 1984) and is an underlying assumption of our discussion.

In addition to the use of anti-peptide antibodies, other functional methods that measure some aspect of antibody–antigen interaction have been used in futile attempts to define protein epitopes. These include:

- absorption of sera with fragments of the antigen,
- antibody protection (of residues against chemical derivatization or peptide bonds against hydrolysis),
- competition tests among monoclonal antibodies,
- reactivity of anti-protein antibodies with variants of the antigen (which include naturally occurring antigenic variants and variants produced by site-directed mutagenesis or selection of escape mutants of viral antigens), and
- molecular modeling (from physical properties of antigens and from energetic analyses of complexes—an approach that has also been used to predict antigenic regions or residues).

Some of the above methods have been partially successful in identification of specific residues or general regions recognized by an antibody, but none has yet allowed the complete definition of an epitope. To date, only determination of the structure of crystals of antibody–antigen complexes has allowed complete definition.

Attempts to match the functional epitope and the structural epitope precisely have not yet been completely successful, although the disparity is not great. Functional assays may indicate that a smaller subset of residues is involved in binding energy, but this probably reflects a limitation on the number of antigenic variants available. In fact, for all the complexes, variants of only a small fraction of the contact residues have been tested. For example, competitive inhibition studies with evolutionary variants of lysozyme correctly identified critical residues in the HyHEL-5 epitope but did not predict its full extent. Similar methods were less successful in predicting the HyHEL-10 contact residues (Lavoie et al., 1990). In addition, all escape mutants that abolish binding of the NC41 antibody to neuraminidase do in fact correspond to residues within the structural epitope. To date, there are no published examples of a mutation of a residue within the structural epitope which does not affect binding.

Molecular modeling has suggested that a subset of residues within the structural epitope may contribute most of the binding energy, i.e., form an "energetic epitope," with surrounding residues allowing structural complementarity (Novotny, 1990). It should be noted that these energetically critical residues are generally not sequentially located. Nevertheless, mutation of the surrounding residues may significantly reduce or even abolish binding. Whether the alteration of binding by changing any given amino acid is due to loss of energy contributing to the interaction by that particular residue, or whether it represents introduction of a "pin" in the interface that interferes with the way the antibody "sits" on the antigen will require refinement of structures of complexes with mutant antigens.

In conclusion, the structural data establish that epitopes on native proteins consist of 15–22 residues in a discontinuous array. Energetic calculations suggest that a smaller subset of 5–6 of these residues contributes most of the binding energy, with the surrounding residues merely indulging in complementarity. It should be stressed that the residues proposed to contribute most of the binding energy are not arranged in a linear sequence but are scattered over the epitope surface; in no sense can they be considered equivalent to unfoldons identified with antisera against short peptides. Ultimately, definition of the precise relationship between the structure and the function of the epitope will require detailed kinetic and structural analysis of site-directed mutants of both antigen and antibody.

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Continuous and discontinuous protein antigenic determinants

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Protein antigenic determinants have been classified as continuous or discontinuous[1,2]. The continuous determinants are composed of residues which are local in the polypeptide sequence, while discontinuous determinants consist of residues from different parts of the sequence, brought together by the folding of the protein to its native structure. Searches made for protein determinants using peptide fragments which compete with protein–antibody complex formation, or peptides that can be used to raise antibodies which crossreact with the native protein, are limited to the simulation of continuous determinants. However, recent experiments[2,3] suggest that most determinants are discontinuous. We now show, by consideration of protein surfaces, that if the recognition zone between a protein and antibody has the same dimensions as those found for the lysozyme–antibody complex[4], none of the protein's surface will be 'continuous'. We suggest that all determinants are discontinuous to some extent, and that crossreacting peptides mimic only the 'primary' interaction site. In addition, we show that the parts of a protein's surface which are most continuous fall predominantly in the loops and/or protruding regions. This explains why quantities such as hydrophilicity[5], accessibility[6], mobility[7] and protrusion[8] can be used to predict which parts of a polypeptide provide the 'best' antigenic peptides.

The surface of a globular protein, as defined by X-ray crystallographic coordinates, is very complex and convoluted. However, visual inspection of any structure shows that most residues on the surface have neighbouring residues that are distant in the sequence. To quantify this observation, we have used the atomic coordinates available from the protein databank[9] to search for 'continuous patches' on the surface of a protein. The method used (see Fig. 1*a*) involves centering a sphere of radius (r) on each surface atom in the protein, and calculating the proportion (F) of the other surface atoms enclosed by the sphere which belong to residues local in the amino-acid sequence. If a sphere encloses only local surface atoms, then we have identified a continuous patch. Surface atoms are defined as those with contact areas (calculated by the method of Lee and Richards[10]) >2 Å^2; local surface atoms are those belonging to residues in the sequence $i-n \rightarrow i+n$, where i is the residue containing the surface atom at the origin of a sphere, and n is an integer in the range 1–10.

When the percentage of surface atoms which lie at the centre of a continuous patch is plotted as a function of sphere radius, we obtain the surface 'continuo-grams' shown in Fig. 1*b*. Four separate plots are presented to show how the results depend on the definition for a local surface atom. These graphs are averaged over three proteins (myoglobin, trypsin and lysozyme), but the individual plots are essentially identical. Note that as the size of the sampling sphere increases, the percentage of the surface which is continuous decreases rapidly, until at a radius of ~10 Å virtually all of the surface is discontinuous, that is, there is no region of 20 Å diameter which contains only atoms from residues local in the amino-acid sequence. This relates directly to the observations made for the lysozyme-antibody complex, where the recognition zone is estimated to be 20 × 25 Å, and is discontinuous[4]. Note however, that this is a monoclonal antibody and has a reasonable affinity for its antigen; the association constant, K_A is in the range $2-4\times10^7$ mol^{-1} (R. Poljak, personal communication). Other antibodies that bind more weakly to their antigens may have smaller combining sites, which might therefore be continuous. As we demonstrate below, however, this is not likely to be the case.

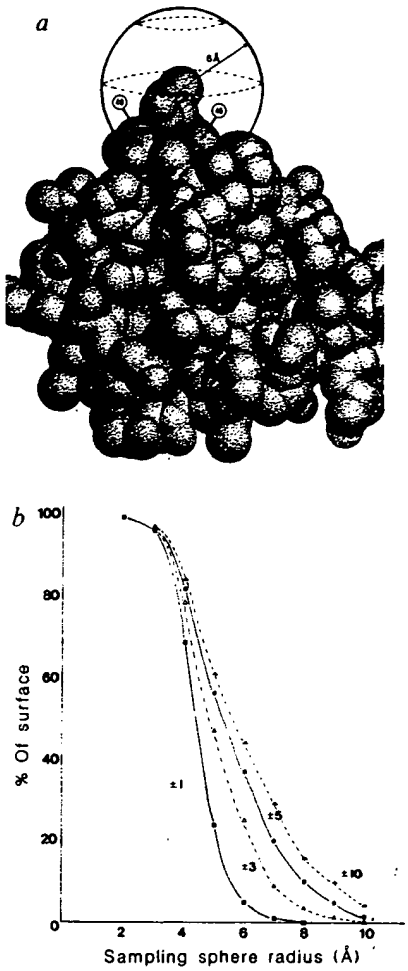

Fig. 1 *a*, Illustration of the method used to calculate the percentage of 'continuous surface' for a protein. The figure shows part of a space-filling model of lysozyme. A sampling sphere of radius 6 Å is centred on one of the surface atoms in the molecule: CG2 of Thr 47. The sphere encloses the atom centres of six other surface atoms, all of which are considered here as local surface atoms, since they belong to residues in the sequence 44–50. Two of the local surface atoms are labelled by residue number. Because all of the atoms enclosed by the sphere belong to local amino-acid residues, CG2 of Thr 47 is considered as the centre of a surface 'continuous patch'. *b*, Surface 'continuo-grams', showing the percentage of surface atoms which lie at the centre of a continuous patch as a function of sampling sphere radius. The four curves are obtained using different definitions for a local surface atom. (Specifically, these are atoms which belong to residues $i-1 \rightarrow i+1$, $i-3 \rightarrow i+3$, $i-5 \rightarrow i+5$, $i-10 \rightarrow i+10$, where i is the residue containing the surface atom at the origin of the sampling sphere.) Note that since the calculations (described in *a* and in the text) consider only atoms whose centres are enclosed by a sampling sphere, these curves represent conservative estimates of the percentage of continuous surface. If the intrusion of any fraction of an atom into the sampling sphere is considered, all of the curves will be shifted to the left.

A low-affinity antibody, with $K_A = 10^4$ mol^{-1}, will have a standard free energy of binding of -6 kcal mol^{-1} (ref. 11). Assuming, therefore, that each Å^2 of the protein surface buried during association contributes 20 cal (ref. 12) to the binding energy, the buried area will be ~300 Å^2. Using sampling spheres of various sizes centred on each of the surface atoms in lysozyme, we calculate that the amount of the protein's surface area would typically involve a patch of ~8 Å radius. As shown in Fig. 1*b*, <10% of the surface patches of this size are continuous. Thus,

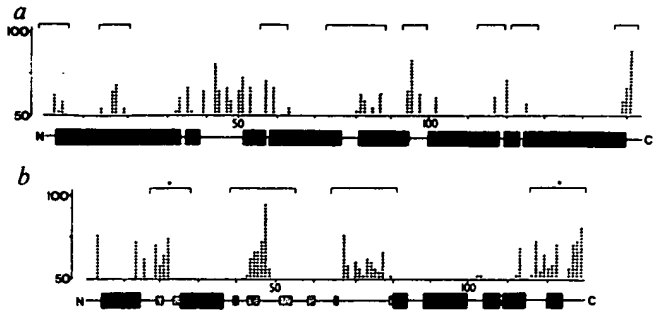

Fig. 2 Variation in $\langle F \rangle$ along the sequence of myoglobin (a) and lysozyme (b). $\langle F \rangle$ values for residues are plotted along the ordinate and the sequences of each protein along the abscissa. The locations of secondary structures in the proteins are shown below the corresponding plot; helices are represented by solid bars, β-strands by stippled bars and loop/turn regions by single lines. The locations of antigenic peptides[13-15] are indicated above each plot; those labelled in b by • are discontinuous.

binding sites even for low-affinity antibodies are unlikely to involve only local amino-acid residues. We conclude that heptapeptides which cross react with protein antibodies mimic only part of the recognition area. Similarly, anti-peptide antibodies which recognize native protein will be limited to surface patches dominated by a short peptide, where interference from non-local residues is minimal.

On this basis, therefore, it would be reasonable to expect that the parts of a protein that are 'most continuous', should provide the best antigenic peptides. As Fig. 2 shows, this is indeed the case; the two plots illustrate the variation in $\langle F \rangle$ along the sequences of myoglobin and lysozyme, where $\langle F \rangle$ represents the mean value of F for all surface atoms in a residue, calculated using a sampling sphere radius of 10 Å (residues which do not contain surface atoms are assigned a value of $\langle F \rangle = 0$). The 'most continuous' patches in each protein are those where the residues have $\langle F \rangle > 50\%$. Note that there is a strong correlation between these regions and the location of antigenic peptides[13-15].

An analysis of the surface compositions of 12 proteins shows that ~50% of all loop residues have $\langle F \rangle$ values >50% (Fig. 3). The corresponding figures for helix and sheet residues are only 23% and 16%, respectively. As would be expected, therefore, most of the residues which form the most continuous regions lie in loops which protrude from the surface of the protein. Some are also found in helices, but none are found in the central strands of a β-sheet (where residues which are distant in the sequence will always be in close proximity).

These results provide a satisfying rationale, taken together with the necessity for antibody accessibilty[6] or protrusion[8], to explain which regions of a protein provide the 'best' antigenic peptides.

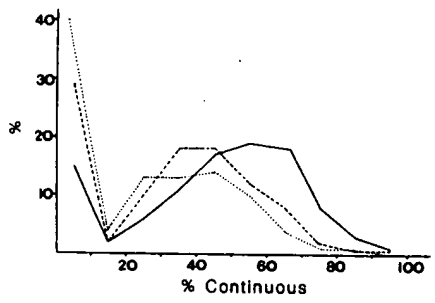

Fig. 3 The distribution of $\langle F \rangle$ values for residues in loop (———), helix (- - - -) and β-strand (· · · · · · ·) regions. % Indicates the frequency of residues with an $\langle F \rangle$ value (% continuous) in a given range.

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Expression of a transfected human c-*myc* oncogene inhibits differentiation of a mouse erythroleukaemia cell line

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The Friend-virus-derived[1] mouse erythroleukaemia (MEL) cell lines represent transformed early erythroid precursors that can be induced to differentiate into more mature erythroid cells by a variety of agents including dimethyl sulphoxide (DMSO)[2]. There is a latent period of 12 hours after inducer is added, when 80–90% of the cells become irreversibly committed to the differentiation programme, undergoing several rounds of cell division before permanently ceasing to replicate[3,4]. After DMSO induction, a biphasic decline in steady-state levels of c-*myc*[5,6] and c-*myb*[6] messenger RNAs occurs. Following the initial decrease in c-*myc* mRNA expression, the subsequent increase occurs in, and is restricted to, the G_1 phase of the cell cycle[7]. We sought to determine whether the down-regulation is a necessary step in chemically induced differentiation. Experiments reported here indicate that expression in MEL cells of a transfected human c-*myc* gene inhibits the terminal differentiation process.

To study the relationship between the down-regulation of the c-*myc* mRNA and cellular differentiation, a plasmid ($PL^1hmcneo^r$) containing the pSV2neo and the human c-*myc* genes was transfected into MEL cells (line 745). The stable G418-resistant transfectants (Fig. 1) were induced with DMSO to determine their differentiation potential. Strikingly, all transfectants which expressed the exogenous human c-*myc* gene failed to develop a red pellet or a positive reaction with benzidine after 7 days of induction. This inhibition of differentiation is not a transfection artefact. The parental MEL cells were subcloned after undergoing a sham transfection without selection, and all 24 subclones examined differentiated after 7 days of DMSO induction. In addition, transfectant T57, which expressed only the neomycin resistance gene, differentiated normally after induction. Introduction of another selection marker, the APRT (adenine phosphoribosyltransferase) gene, also does not interfere with induced differentiation of MEL cells[8].

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THE ANTIGENIC SURFACE OF STAPHYLOCOCCAL NUCLEASE

II. Analysis of the N-1 Epitope by Site-Directed Mutagenesis[1]

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Previous studies in our laboratory on the production and isolation of a panel of mAb to staphylococcal nuclease allowed us to define a series of eight overlapping epitopes. Using site-directed mutagenesis of the nuclease coding sequences we were able to map the nonoverlapping epitopes recognized by two members of this panel. In the study reported here, we report the generation and analysis of a number of single amino acid substitutions for seven surface residues predicted to lie within one of these two epitopes. Immunochemical analysis showed that one or more substitutions at each of these seven positions had a major effect on mAb binding, whereas other substitutions had none. Based on the nature of these substitutions and the chemical and physical properties of the variant molecules, we believe that any structural effects induced by these substitutions are local and do not result in long-range structural alterations that indirectly influence antibody reactivity. Therefore, we conclude that disruption of mAb binding can be directly attributed to changes in amino acid side chains and that not only are all seven of the residues studied part of the epitope but all seven make contact with the antibody combining site. These studies demonstrate the advantages of using site-directed mutagenesis to study antigen structure and emphasize the importance of constructing and examining multiple substitutions for any given amino acid.

Examination of the reaction between Ab[3] and members of a panel of closely related, homologous proteins has been one of the most successful approaches to the study of protein Ag structure (1). The data obtained were a correlation between differences in amino acid sequence among the proteins and their reactivity with Ab. Such studies led to the localization of multiple epitopes on a number of Ag and to the unambiguous demonstration of the presence of discontinuous epitopes on the surface of protein Ag. Of particular note was the accurate prediction of the HyHEL5 epitope on the surface of HEL (2); a model essentially confirmed in most essentials upon solution of the crystal structure of the HEL-HyHEL5 complex by Sheriff et al. (3).

Despite these successes, this approach is severely limited by the number of naturally occurring variants and even more so by the number of available variants with different amino acids at the same position within the epitope. The ability to ask other questions about the Ag-Ab interaction, such as the nature of the interface formed by Ag and Ab, or the conformational effects of this interaction is similarly limited by the number of variant Ag molecules available and by the extent of difference between any two Ag being compared. For example, differences in reactivity of an Ab with two or more Ag may not be assigned to particular residues because in each case there is more than one difference between the two Ag within the epitope. Similarly, the absence of major conformational changes in HEL upon interaction with the HyHEL5, HyHEL10 or D1.3 mAb (3–5) may be due to structural rigidity imposed on the HEL molecule by its four intrachain disulfide bonds necessitating examination of the structure of complexes of mAb and non-disulfide-bonded protein Ag.

To bypass many of these limitations and to begin to answer some of these questions, it is necessary to develop an alternative Ag-Ab system for which the Ag lacks disulfide bonds, for which the crystal structure is known to high resolution, and for which the coding sequences have been cloned into a high level expression vector to permit the easy manipulation of coding sequences. Systematic manipulation of the coding sequences would allow isolation of a large panel of single amino acid variants of the Ag and high level expression would allow production of sufficient material for complete immunochemical, chemical, and physical analysis.

In the accompanying report (6) we reported the initial development of such a system with NASE as the selected Ag. We also reported the production and properties of a panel of anti-NASE mAb that defines a series of eight overlapping epitopes. In addition, we showed that site-directed mutagenesis of the NASE coding sequence resulted in single amino acid variants that allowed localization of several epitopes on the NASE surface. In this communication, a more detailed analysis of one of these epitopes is reported.

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[3] Abbreviations used in this paper: Ab, antibody; NASE, staphylococcal nuclease; HEL, hen egg-white lysozyme; CD, circular dichroism.

MATERIALS AND METHODS

Isolation of NASE from pFOG405. Escherichia coli K-12 containing the NASE-encoding plasmid pFOG405 (7) was grown overnight in LB medium supplemented with 0.08% glucose and 25 to 50 μg/ml ampicillin. The culture was diluted 50× into complete MOPS medium and after incubation with shaking at 37°C for 5 to 8 h, NASE was isolated according to Serpersu et al. (8) and further purified by FPLC (Pharmacia Fine Chemicals, Piscataway, NJ) cation exchange chromatography using a simple 0 to 0.25 M NaCl linear gradient in 0.05 M sodium phosphate buffer, pH 8.5.

Oligonucleotide mutagenesis. A series of single amino acid variants of NASE was generated by oligonucleotide-directed mutagenesis using the method of Kunkel and co-workers (9, 10) as described in the Bio-Rad publication 170-3571 (Bio-Rad Laboratories, Inc. Richmond, CA). Oligonucleotides were synthesized at the Protein and Nucleic Acid Research Facility at the University of Virginia. Mutants were identified by DNA sequence analysis and the mutated sequence substituted for the native sequence in the pFOG vector. Variant NASE was expressed and the protein purified as described above for the native protein. The resulting proteins are referred to by the single letter amino acid code[4] for the wild-type protein, the position in the primary amino acid sequence of that amino acid, followed by the variant amino acid in single letter code, e.g., K53N is an asparagine (N) substitution for a lysine (K) at position 53.

Competitive inhibition assay. The relative ability of variant Ag to interact with mAb was determined by competitive inhibition in a modified ELISA assay as follows: Ag at varying concentrations was mixed with Ab to give a final Ag concentration of 0.1 to 100 μg/ml. The Ab concentration was predetermined to give a known endpoint OD_{414nm} in the ELISA assay in the absence of any inhibitor. All Ag and Ab solutions were prepared in PBS containing 0.1% BSA, and these mixtures were allowed to equilibrate overnight at 4°C. These inhibitor Ag-Ab mixes were each pipetted, in triplicate, into wells of a 96-well ELISA assay plate that had been precoated with native NASE at 1.0 μg/ml. These plates were then incubated at 37°C for 1 h and subsequently processed as for the standard ELISA assay. Positive and negative controls were run with and without added native NASE as inhibitor. Results are expressed as the percent inhibition of the reaction of mAb in the absence of any inhibitor.

Enzyme activity. Enzyme activity was determined by the hyperchromicity assay of Cuatrecasas et al. (11) at 37°C in 25 mM Tris HCl, pH 8.8, 10 mM $CaCl_2$, and DNA at 50 μg/ml. A unit of enzyme (the amount of enzyme required to cause an increase in absorbance of 1.0/min) was determined from a plot of initial rate (V) vs amount of enzyme used. The specific activity of NASE was then calculated as U/mg enzyme. K_m and V_{max} were determined from double reciprocal plots of 1/(V) vs 1/(S) in similar assays using a constant amount of NASE (50 ng) and varying amounts of DNA (10 to 40 μg) in a final volume of 1.0 ml.

Physical analyses. Affinity constants were estimated by a modified ELISA assay according to Friguet et al. (12) with the correction described by Stevens (13) for whole antibody.

Circular dichroism (CD) spectra were obtained for purified NASE and selected NASE variants using a Jasco J-600 spectropolarimeter at room temperature with a 0.1-cm path length. Samples were at approximately 100 μg/ml in 0.05 M sodium phosphate buffer, pH 8.5, containing 0.06 M NaCl. Molecular ellipticity (θ) (in deg. cm^2 $decimol^{-1}$) was calculated and plotted vs wavelength (λ).

Tryptophan fluorescence emission spectroscopy was performed using a Perkin-Elmer LS-5 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). Samples were prepared in 25 mM Tris, pH 8.8, 10 mM $CaCl_2$ buffer (Tris/$CaCl_2$) and diluted to give equivalent absorbance at 280 nm, typically A_{280} = 0.1. For fluorescence emission measurements the samples were excited at 280 nm and the resulting emission was scanned from 300 to 400 nm. Acrylamide quenching of fluorescence was determined by adding increasing amounts of 6 M acrylamide, dissolved in the same buffer, to final concentrations of 0.1 to 0.5 M. For the quenching experiments, samples were excited at 295 nm and the emissions were scanned from 310 to 400 nm. The quenching constant was calculated from the slope of a Stern-Volmer plot as described by Leto and Holloway (14).

[4] The single letter code for amino acids used in this paper are as follows: A, alanine; E, glutamic acid; F, phenylalanine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine; W, tryptophan.

RESULTS

Modeling of epitope N-1. The epitope, herein referred to as the N-1 epitope, defined by mAb-1 and localized using the K133T variant NASE molecule (6) was selected for further study because K133 lies on a relatively flat surface of NASE (and, in that sense, corresponded to the three HEL epitopes for which the crystal structure is known) and because the sole tryptophan residue of NASE (W140) is predicted to lie within the N-1 epitope (see below). W140 has numerous contacts with K133 (the position used to originally localize the epitope) and is an excellent reporter molecule for physical studies.

The topography of NASE, in the vicinity of K133, was examined on a Silicon Graphics Iris System (Silicon Graphics, Mountain View, CA) using atomic coordinates taken from the Brookhaven Protein Data Bank (Brookhaven National Laboratory, Upton, NY) (Fig. 1). Based on the known dimensions and other properties of the three known HEL epitopes (3–5), the N-1 epitope was predicted to lie on a relatively flat surface with outside dimensions of approximately 25 × 25Å covering about 750$Å^2$ solvent accessible surface area. As such it was predicted to consist of 10 core residues (Y54[5], K110, V111, A112, E129, K133, K134, K136, I139, and W140) and seven boundary residues (E52, K53, Y113, V114, R126, A130, and S141); the terms core and boundary being used merely to indicate position within the epitope. These boundary residues may or may not contact mAb-1. A130 and S141 were assigned to the boundary because their side chains point away from the selected surface. The positions of residues 142–149 have not been determined in the crystal structure, indicating the extreme mobility of this region of NASE, and none of these residues has been predicted to be within the epitope. A truncated molecule terminating at S141 is being constructed to test this possibility. Although other epitope structures, which would also contain residue 133, are possible the N-1 epitope predicted above is merely a working model and as such is an experimentally testable hypothesis.

Competitive inhibition assays. These studies focused on seven of the core residues—K133 and six other residues surrounding it (Fig. 1). One or more single amino acid variant molecules for each of these positions was produced and examined for their ability to inhibit the reaction between NASE and mAb-1. The results for variants at position 129 and 133 are shown in Figure 2.

The K133 variants could be placed into two distinct groups: 1) those which were immunochemically identical or very similar to native NASE and which retained a positively charged side chain, i.e., the K133R and K133H variants; and 2) all other variants studied each of which required approximately 100-fold more Ag than native NASE to give 50% inhibition. All variants at position 133 were equivalent to native NASE in their reactivity with mAb-25 that defines the N-25 non-overlapping epitope on NASE (6). As another control the H46R variant that localizes the N-25 epitope, reacts the same as native NASE with mAb-1 whereas showing the expected reduced reactivity with mAb-25 (Fig. 2).

In contrast, variants at position 129 of NASE showed a broader range of reactivity with mAb-1 whereas maintaining normal reactivity with mAb-25 (Fig. 2). For ex-

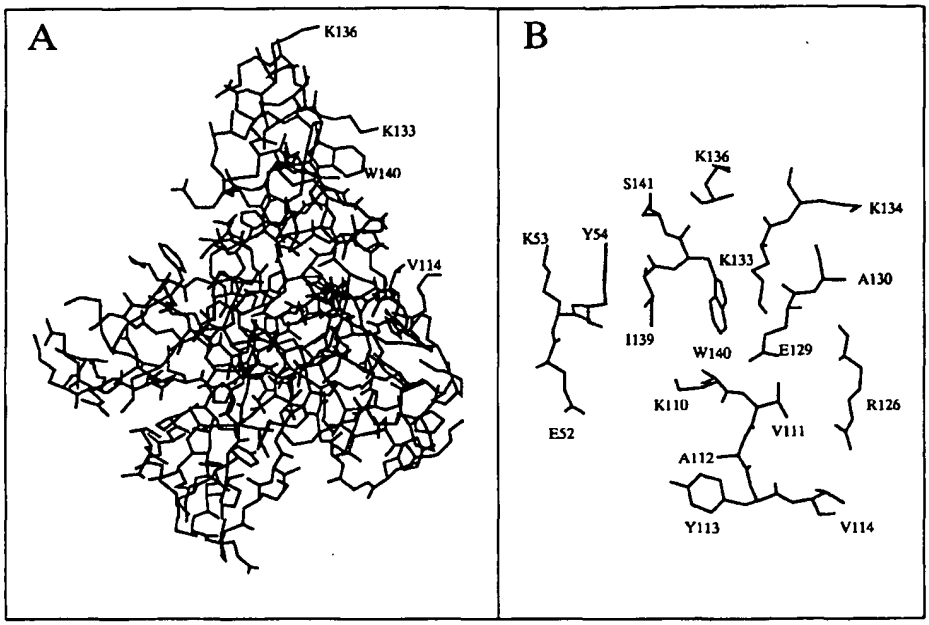

Figure 1. Graphic representation of staphylococcal nuclease and the N-1 epitope. *A*, The complete structure of NASE with the lysine at position 133 (K133) and its adjacent tryptophan at position 140 (W140) indicated. The proposed N-1 epitope lies along the side of the NASE molecule with residues K136 and V114 at the top and bottom as shown. *B*, Frontal view of the surface amino acids predicted to be within the N-1 epitope. The residues studied in this report are K110, E129, K133, K134, K136, I139 and W140.

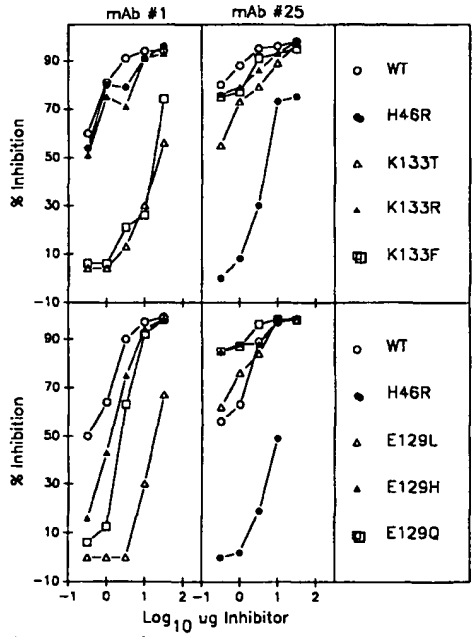

Figure 2. Inhibition of mAb-1 and mAb-25 binding to native NASE by variants at positions 133 and 129. Varying amounts of variant or native NASE was added to an amount of mAb predetermined to give a set endpoint in a standard ELISA assay. After incubation, aliquots were added to triplicate wells of a microtiter ELISA assay plate that had been precoated with native NASE. The amount of mAb that bound to the assay plate was subsequently determined by a standard ELISA assay. The extent of inhibition was calculated and plotted vs the amount of inhibitor added.

ample, the E129L variant required at least 100-fold more Ag, than did native NASE, for 50% inhibition whereas the E129H and E129Q variants had intermediate effects.

The results of competitive inhibitions using variants at each of the five other positions studied are shown in Table I. It is quite clear that one or more substitutions at each of the seven core positions tested have major effects on binding by mAb-1, e.g., the K110S and K136N variants. It is also clear that some substitutions had little or no effect, e.g., the K110Q and I139V variants. In all cases reactivity with mAb-25 was essentially the same as that seen with native NASE.

TABLE I
Summary of competitive inhibition analysis

Amount of Ag[a]		Inhibiting Ag[b]
≤1 μg/ml	NASE	K134L (3), K134T (3)
	K110Q (3)	I139V (1)
	E129H (3)	W140L (1), W140S (1)
	K133H (3), K133R (1)	
>1 μg/ml	K110T (10), K110A (10)	K136N (33)
	K110S (30)	I139K (10), I139M (10)
	E129Q (17)	W140Y (50), W140K (30)
	K134N (10)	
>10 μg/ml	K110H (100)	K133T (83), K133F (83)
	E129L (67)	K133L (116), K133Y (200), K133N (83)

[a] Amount of antigen required for 50% inhibition.
[b] Numbers in parentheses are estimates of the relative amount of variant protein required to give inhibition equivalent to native Ag.

As a final control, the reactivity of a number of these variant molecules with other mAb, each belonging to a complementation group different from that of mAb-1, was tested. In all cases the reactivity of the variants was equivalent to that observed with native NASE (data not shown).

Enzyme activity. The enzymatic activity of native NASE and several variants was determined in an initial attempt to assess the effect of the amino acid substitutions on the structure and function of NASE. Some of the results of these studies are shown in Table II. The K133R variant, which reacted normally with mAb-1, had native enzyme activity. In contrast, substitution of threonine for lysine at this same position (the K133T variant), a change that dramatically affected reactivity with mAb-1, resulted in reduced enzyme activity. Other variants, including H46R that has no effect on mAb-1 binding, also had reduced enzymatic activity.

H46R showed a native K_m and a reduced V_{max}, consist-

TABLE II
Enzyme activity values for NASE and selected variants

Enzyme	SA[a]	V_{max}	K_m
NASE	2,558	4.51	19
H46R	570	1.43	19
K133R	2,546	3.80	20
K133T	999	5.12	50

[a] Specific activities (SA) of the enzymes were determined from the initial rate of DNA hydrolysis and are given in Units/mg protein. The V_{max} and K_m values were determined from Lineweaver-Burke plots. The units for V_{max} are $\Delta Ab\ min^{-1}\ \mu g^{-1}$ and those for K_m are $\mu g/ml$.

ent with the position of this residue on the lip of the catalytic site. The K133T variant showed a native V_{max} and a reduced K_m indicating a reduced affinity for the DNA substrate. In contrast, substitution of arginine for lysine at position 133 (the K133R variant) had little or no effect on K_m or V_{max}. These results could be explained either by a conformational change induced by the lysine to threonine substitution which affected both enzyme activity and reactivity with mAb-1 or by removal of a positive charge leading to reduced affinity for the DNA substrate without conformational change and to reduced binding to mAb-1.

Physical analyses. The association constants (K_A) for mAb-1 and mAb-25 binding to several of the variant Ag were estimated by a modification of the ELISA-assay (12, 13). Variants with similar inhibition patterns, e.g., K133R and H46R, bound each antibody with an affinity similar to that of native NASE whereas those variants with reduced reactivities bound antibody with a reduced affinity (data not shown).

The normal reactivity of all variants, at these seven positions, with all other mAb tested suggested that any conformational effects that may have occurred were localized to the immediate vicinity of the altered residue. However, the reductions in enzymatic activity seen with some variants at positions apparently not involved in catalysis suggested that long range effects may be occurring. In an attempt to determine what was responsible for reduced enzyme activity, native NASE and selected variants were subjected to CD and tryptophan fluorescence emission analyses as independent assessments of structural alterations.

The CD spectra for native NASE and the K133T and H46R variants are shown in Figure 3. Each spectrum represents the mean of five measurements on the same sample. It is clear that the spectra of the variant NASE molecules are essentially identical to that for native NASE. Any differences seen are well within the variations observed between different samples prepared from the same stock solutions and/or different preparations of the same protein. These results suggest there are no gross structural differences between variant and native NASE molecules.

In native NASE the sole tryptophan residue (W140) is in extensive van der Waals contact with the side chain of lysine at position 133 (K133) (Fig. 1). These interactions are thought to stabilize the tryptophan hydrophobic side chain on the surface of NASE in an aqueous environment. As a further assessment of whether structural changes were induced by the amino acid substitutions made, tryptophan fluorescence emission analyses of NASE and selected variants was performed. The UV absorption spectra for the variant molecules did not differ

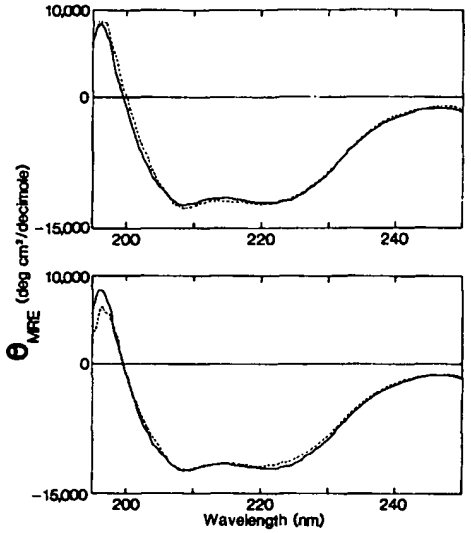

Figure 3. Circular dichroism spectra of native NASE and selected variants. The spectrum for native NASE is the *solid line* in each panel. The spectrum for the K133T variant (*top*) and H46R variant (*bottom*) are the *dashed lines*. The *ordinate* is the mean molecular residue ellipticity in degrees cm^2/decimol. Spectra are the average of five scans on a JASCO J600 spectrometer using a 0.1-cm path cuvette. Protein concentrations were approximately 100 $\mu g/ml$ in 0.05 M Na_2PO_4/0.06 M NaCl, pH 8.0.

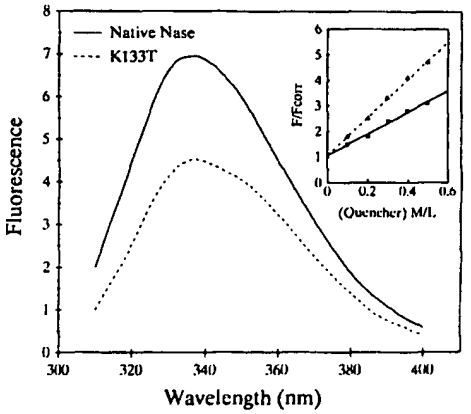

Figure 4. Tryptophan fluorescence emission. The tryptophan fluorescence emission spectra for native and variant NASE were determined at equivalent concentrations in a Perkin-Elmer LS-5 fluorescence spectrophotometer. Samples were prepared in 0.025 M Tris.HCl/0.01 M $CaCl_2$, pH 8.8. Samples were excited at 280 nm and emission scanned from 300 to 400 nm. The insert shows the results of acrylamide quenching experiments. Samples were excited at 295 nm and emissions scanned from 310 to 400 nm. The peak fluorescence emission in the presence of various amounts of acrylamide was determined and the results are presented as Stern-Volmer plots (14). Shown are the results for native NASE (*solid line*) and the K133T variant (*dashed line*).

from that of the native NASE molecule (data not shown). Some variants showed decreased fluorescence emission, e.g., K133T and K133R, whereas others did not, e.g., E129Q and K133N. In no case was there a change in the peak emission wavelength and there was also no correlation between extent of emission and reactivity with mAb-1. For example, both the K133T and K133N variants show the same reduced reactivity with mAb-1 and normal reactivity with all other mAb tested. However, the K133T variant showed a decreased fluorescence emission and increased quenching by acrylamide (Figure 4), whereas the K133N variant showed normal fluorescence and quenching (data not shown). In each case where

decreased fluorescence emission was observed there was also demonstrable increased quenching of fluorescence by acrylamide (Fig. 4). These results are consistent with the absence of structural alterations in the tryptophan environment of the variant molecules except for increased exposure of the tryptophan side chain to solvent. The fluorescence emission spectra and Stern-Volmer quenching plots for native NASE and the K133T variant are shown in Figure 4.

DISCUSSION

Site-directed mutagenesis was used to generate a series of genetic variants of NASE with single amino acid substitutions at each of seven surface residues predicted to lie within the N-1 epitope recognized by the mAb-1 anti-NASE antibody. These residues include the amino acid that originally mapped the epitope (K133) (6), the sole tryptophan (W140) with which K133 has extensive van der Waals contacts, and five other residues that form a ring around these two residues. Immunochemical analysis of the reaction of mAb-1 with native NASE and 26 variant molecules showed that one or more substitutions at each position had a major effect on mAb-1 binding. Other substitutions at six positions had no effect; the only exception being position 136 for which only one variant was available. In conducting our studies we assumed that an amino acid is part of the epitope if any substitution resulted in significantly decreased binding of antibody in the absence of demonstrable gross structural effects. This assignment does not define the residue as a contact or non-contact residue. A residue is defined as a contact residue if the substitution leading to decreased binding does not significantly increase the side chain volume. Substitution of a bulky side chain for that of a smaller non-contact residue could alter binding by preventing antibody from establishing normal bond distances with other contact residues. In assessing the contribution of an amino acid to the epitope, using any method short of crystallographic analyses, one is limited to studying the role of side chain atoms. Therefore, the absence of any effect on Ab binding to any given variant does not rule out significant participation of one or more main chain atoms of that particular residue. Following these guidelines, we consider all seven residues studied to be contact residues.

Of particular interest was the observation that the K133R and K133H variants were immunochemically indistinguishable from native NASE. In contrast, any other substitution at position 133 drastically reduced binding. This strongly suggests that a positive charge at this position is critical to binding and that perhaps the lysine residue in native NASE forms a salt bridge with a negatively charged amino acid(s) in the antibody combining site. Although only three changes were examined at position 129, the current results suggest that a polar residue may be required because substitution of histidine or glutamine for glutamic acid at this position had an intermediate or no effect on mAb-1 binding whereas substitution of valine had a major effect. Study of other changes at this residue is required before any substantive conclusion can be made.

An important assumption underlying this analysis is that the effect of the changes in the variant molecules are local to the epitope and are not long range conformational effects that indirectly influence antibody binding. We do not think that non-specific, long range structural alterations explain the decreased immunochemical activity of many, if any, of these variants for the following reasons. 1) All variants reacted normally with all other mAb including mAb-25 which is sensitive to the change in the H46R variant. Collawn et al. (15) have reported that mAb can be sensitive to subtle conformational changes at sites distal to the region recognized by the antibody. Therefore, we believe the native reactivity of all variants with the other anti-NASE mAb strongly supports the absence of long-range conformational affects. 2) All variants examined were identical to native NASE by circular dichroism spectroscopy (which is a standard measure of protein secondary structure) with the CD spectra showing the characteristic ellipticity minima at 208 and 222 nm, consistent with published data (16). 3) The tryptophan fluorescence emission spectra indicate that the variants at position 133 and 129 do not cause a change in the environment of the tryptophan at position 140. We considered the possibility that changes at position 133 disrupted mAb 1 binding by reducing side chain interactions between W140 and the residue at position 133, placing the W140 residue in a more aqueous, and thus thermodynamically less favorable, environment. If true, then one might have expected the W140 side chain to attempt "burying" itself on the surface of the protein, thereby inducing significant local conformational changes. However, if the W140 side chain in the variants was attempting to bury itself, then one might have expected to see a shift in its fluorescence emission maximum. The results of the fluorescence emission spectroscopy studies presented above show no such shift. Rather, they show a quantitative decrease in emission, suggesting increased accessibility of the indole ring of W140 to solvent quenching as one might expect from a change in van der Waals contacts between W140 and the amino acid at position 133 in these variants. This conclusion is supported by the acrylamide quenching experiments reported above including the observation that the K133N variant, although substantially reduced in immunochemical reactivity, showed a normal emission spectrum with native quenching by acrylamide. 4) Although some variants had lower enzymatic activity, there was no correlation between the level of enzyme activity and immunochemical reactivity with any mAb. There was a correlation of immunochemical and enzymatic activities with the retention of a positive charge at position 133. This correlation, however, may be more apparent than real in the sense that the positive charge may be involved in binding of negatively charged DNA substrate (17) and coincidentally, but independently, involved in a salt bridge with residues in the antibody combining site. This conclusion is supported by the observation that the K133R variant has normal enzyme sp. act., K_m and V_{max} whereas the K133T variant had a decreased specific activity and normal V_{max} but an increased K_m. Thus the loss of enzymatic activity by the K133T variant correlates with a decreased affinity for substrate. In addition the I139M change had much less an effect on antibody binding and a much greater effect on enzyme activity than

did the K133T substitution. The mechanism of catalysis and substrate binding by NASE has been analyzed using the inhibitor thymidine-3',5'-diphosphate, and whereas it has been postulated that the binding of the inhibitor is an appropriate model for DNA binding, the role of residues outside the active site has not been adequately addressed (18).

Therefore, we believe the observed physical and chemical properties of the NASE variants reported here support our conclusion that the disruption of mAb-1 binding can be directly attributed to changes in the side chains rather than to global structural effects, implicating these seven residues as contact residues within the epitope. This conclusion is also supported by the studies of others that suggest alterations in surface residues have only local effects (19, 20).

Our results emphasize the importance of individual amino acids to the antigenicity of a specific epitope as has been observed in other systems (1–5). They also demonstrate the advantages of using site-directed mutagenesis to study Ag structure and the contribution of individual amino acids to reaction with antibody. It is apparent that our approach has released us from the limitations seen in early epitope mapping experiments using homologous proteins and emphasizes the importance of constructing and examining more than one variant at each position. Not all substitutions alter reactivity and the use of only a few variants may prohibit assignment of any given amino acid to the epitope much less its designation as a contact residue. Similarly, assignment of a critical and/or primary role for any given residue based solely on the effect of one or, at most, a few differences between any two or more Ag would be inappropriate without a more detailed knowledge of the nature of the other contact or buried residues. Several studies have reported the use of site-directed mutagenesis in the study of mAb-Ag interaction (21). In most cases, however, the studies were intended only to localize epitopes on the surface of a protein Ag and few changes were made—certainly not many at any given residue—with no independent analysis of structural integrity.

Site-directed mutagenesis has allowed us to generate a larger panel of single amino acid variants at a single site than exists for any other model Ag system for all sites. We have demonstrated that conclusions concerning the most likely nature of the contacts between a given residue and the antibody combining site may be obtained from a thorough analysis of multiple variants at a given position. We have also demonstrated that alterations in surface residues have only local effects on protein structure. We are continuing our analysis of the N-1 epitope as well as other epitopes defined by other mAb. Of particular interest is the apparent fine specificity differences noted for mAb-25 and mAb-19 that belong to the same complementation group but show different reactivity with the H46R variant (6). Also of significant interest is a study on the structural basis for overlapping epitopes as defined by competitive inhibition analyses (6, 21).

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THE ANTIGENIC SURFACE OF STAPHYLOCOCCAL NUCLEASE

I. Mapping Epitopes by Site-Directed Mutagenesis[1]

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The analysis of the antigenic surface of staphylococcal nuclease was begun by generating and characterizing a panel of mAb. Twelve mAb were selected from a large number of anti-nuclease mAb and characterized for affinity and isotype, by their ability to block enzyme activity, and by complementation and competitive inhibition assays for the relative location of epitopes. The mAb were placed in complementation groups based on their distinct binding patterns. These groups define a series of eight overlapping epitopes that are estimated to cover a large portion of the nuclease surface. Four mAb blocked the enzyme activity of nuclease. The epitopes defined by two of these four mAb were localized on the surface of nuclease using single amino acid variant Ag generated by site-directed mutagenesis of the cloned nuclease coding sequence. mAb-25 maps to residue 46 which is located at the edge of the enzyme active site consistent with its ability to inhibit enzyme activity. mAb-19, which also blocks enzyme activity and belongs to the same complementation group as mAb-25, was unaffected by the the substitution at position 46. This suggests that mAb-19 and mAb-25, if they do react with the same epitope, have differences in fine specificity. mAb-22 blocks enzyme activity and belongs to an overlapping complementation group. The fourth mAb, mAb-1, which belongs to a distinct, nonoverlapping, complementation group, does not blocks enzyme activity, and is directed to a region of nuclease that includes the amino acid at position 133. This residue is located a short distance from the active site in a region that has been suggested to participate in binding of DNA, a substrate for nuclease. Therefore, the four epitopes defined by these mAb are localized at or near the enzyme active site.

Previous studies on the molecular basis of protein Ag structure have focused on the use of chemical alteration of amino acid side chains (1, 2), generation of fragments of protein Ag (3, 4), synthetic peptides (1, 5) and the use of naturally occuring genetic variants of the Ag (6, 7). Although these approaches have yielded valuable information, each has been limited in that either only a few amino acids within the epitope could be identified or a single segment of a multi-segment epitope could be studied. In the study presented here and in the accompanying paper (8), we describe a system for studying epitope structure and Ag-antibody interactions that permits full genetic manipulation of the protein Ag structure. This in turn, allows the determination of the contribution of any amino acid to the binding of Ag by antibody.

We have chosen NASE[3] (ribonucleate (deoxyribonucleate)-3′-nucleotidohydrolase, EC3.1.4.7) as our model Ag because it is structurally well defined and because the cloned gene (9) in a high level expression system made genetic manipulation and isolation of large amounts of mutant antigen feasible. In this report, we describe the generation and characterization of a panel of monoclonal antibodies to NASE and the use of a limited number of single amino acid variants to map several epitopes. In the accompanying report (8), we describe the generation of a series of single amino acid mutant NASE molecules used to more fully characterize one epitope.

MATERIALS AND METHODS

Animals. CAF_1 and BALB/c mice were obtained from The Jackson Laboratories, Bar Harbor, ME and Flow Laboratories, McLean, VA respectively. The mice were hyperimmunized with NASE as previously described for human serum albumin (10) and their spleens removed 4 days after the last injection for fusion.

Ag. NASE was initially purchased from Sigma Chemical Co., St. Louis, MO and used without further purification. It was later produced in *Escherichia coli* using the pFOG405 expression vector (11). Preliminary studies showed the recombinant NASE to be immunochemically indistinguishable from NASE isolated from cultures of *Staphylococcus aureus* (Foggi strain).

Fusion. Hybridomas producing anti-NASE monoclonal antibodies were generated, in the Lymphocyte Culture Center at the University of Virginia, by fusion of spleen cells with SP2/0-Ag.14 myeloma cells using the method of Kohler and Milstein (12) as previously described (13). Culture supernatants were screened by a direct-binding ELISA assay and hybrids producing anti-NASE antibodies were cloned by limiting dilution. Selected hybrids were expanded in culture for production of ascites fluid and for cryopreservation.

Antibody production and purification. Pristane-primed BALB/c mice were injected with 1×10^7 hybridoma cells i.p. Ascites fluid was collected and the lipids removed by precipitation with dextran sulfate and $CaCl_2$. The clarified ascites fluid was then diluted 1/1 (v/v) with 0.15 M NaCl and the Ig precipitated with an equal volume of ice-cold saturated ammonium sulfate. The precipitated Ig was dissolved and dialyzed against PBS and subsequently affinity puri-

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[3] Abbreviations used in this paper: NASE, staphylococcal nuclease; HRPO, horseradish peroxidase.

fied by protein-A chromatography.

This purified antibody was then conjugated either with HRPO (type V, Sigma) according to Farr and Nakane (14) or with biotin using the Enzotin reagent (ENZO Biochem., New York). In the case of the biotin-conjugated antibodies, HRPO-conjugated strepavidin (Sigma) was used in the ELISA assay. No significant differences were found using either reagent.

Antibody H and L chains were isotyped in an ELISA trapping assay using microtiter plates coated with goat anti-mouse Ig. Culture supernatants were added and the assay developed with HRPO-conjugated, isotype-specific, rabbit antisera (HyClone Laboratories, Inc., Logan, UT).

Complementation and competitive inhibition assays. ELISA-based complementation and competitive inhibition assays were performed to determine the relative location, on the surface of NASE, of the epitope recognized by each mAb.

The complementation assay was carried out essentially as described by Smith-Gill et al. (15). Microtiter plates were coated with unlabeled anti-NASE mAb. After incubation, blocking of unreacted sites and washing, purified NASE was added to each well. After another incubation and washing, another HRPO-conjugated (or biotin-conjugated) monoclonal anti-NASE was added. The ability of the second anti-NASE antibody to bind to the immobilized NASE was assessed by addition of substrate.

The competitive inhibition assay was performed as previously described (13). HRPO-conjugated (or biotin-conjugated) anti-NASE mAb was mixed with varying amounts of unlabeled antibody. Immediately thereafter an aliquot of this mixture was added to wells of a microtiter plate that had been coated with NASE. After incubation and washing, the substrate was added and the amount of antibody bound was determined. The extent of inhibition was determined relative to inhibition with an equivalent amount of the same or an irrelevant antibody.

Determination of K_A. Antibody binding constants were determined by ELISA as described by Friquet et al. (16) using the calculation correction for whole antibody described by Stevens (17). The K_D was determined as the slope of a plot of the reciprocal of free antibody at equilibrium vs the reciprocal of the total Ag concentration. The K_A was calculated as the reciprocal of K_D.

Enzyme activity. Enzyme activity was determined by monitoring the increase in absorbance at 260 nm of boiled calf thymus DNA after the addition of an aliquot of purified NASE enzyme as described by Cuatrecasas et al. (18). The change in absorbance was monitored on a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) and the initial rate of hydrolysis determined using a resident kinetics program. One unit of activity is defined as the amount of protein required to cause a change in absorbance of 1.0. The sp. act. is defined as U/mg enzyme.

The effect of antibody on enzyme activity was determined using the same assay with a constant amount of enzyme and various amounts of antibody. The initial rate of enzyme reaction under these conditions was determined as above.

Antibody binding to antigenic variants. Antigenic variants of NASE were generated by site-directed mutagenesis as described in the accompanying report (8). The recombinant variants were cloned into the pFOG405 expression vector and expressed in *E. coli*. Recombinant NASE and recombinant variants were purified by FPLC on a Mono-S ion-exchange column using a 0 to 0.25 M NaCl gradient in 0.05 M sodium phosphate buffer, pH 8.5. These Ag were used to coat wells of a microtiter plate in a direct-binding ELISA assay with the anti-NASE mAb.

RESULTS

Characterization of anti-NASE mAb. Two fusions were performed; one with CAF_1 spleen cells and one with BALB/c spleen cells. From a large number of anti-NASE positive clones, 25 were selected for further study. Antibody purified from ascites fluid was used in complementation and competitive binding assays, as described above, to determine the relative location of the epitopes defined by each mAb. Typical results of the complementation assay are shown in Figure 1*A* in which the results are presented as percent maximum binding, setting the highest absorbance value in each assay as equal to 100%. It is clear that these four antibodies define three independent, nonoverlapping epitopes and one epitope that "overlaps" with two others. For example, mAb-6 binds to trapped Ag regardless whether mAb-1, mAb-10, or mAb-12 was used as the trapping antibody. Thus mAb-6 defines an epitope that is spatially independent of the other three epitopes. mAb-12 does not bind to mAb-1 trapped Ag whereas mAb-6 and mAb-10 do bind. Similarly, mAb-12 does not bind well to mAb-10 trapped Ag whereas mAb-1 and mAb-6 bind very well. Thus, the epitope defined by mAb-12 "overlaps" both the epitopes defined by mAb-1 and mAb-10. However, mAb-1 does bind to mAb-10 trapped Ag and mAb-10 binds to mAb-1 trapped Ag consistent with each of these antibodies defining separate, nonoverlapping epitopes.

To determine whether the results of the complementation assay may have been caused by inhibited access to antibody-immobilized Ag, a competitive binding assay was set up in which varying amounts of one unlabeled mAb were mixed with a constant amount of a second labeled mAb before addition of the mixture to assay plates containing immobilized NASE. The results (Fig. 1*B*) obtained by this assay were identical to those obtained in the complementation assay described above, e.g., mAb-12 blocks binding of both mAb-1 and mAb-10 that do not block the binding of each other, etc. Again, these results are consistent with the presence of three spatially separate epitopes (defined by mAb-1, mAb-6, and mAb-10) and a fourth epitope (defined by mAb-12) that "overlaps" two of them.

Similar complementation and competitive binding assays were conducted with the full group of 25 antibodies. Based on the results of these assays, a panel of eight mAb was selected that together define eight complementation groups (or eight overlapping epitopes) (Table I; Fig. 2). Another four mAb were selected as additional members of four of these complementation groups.

The H and L chain isotypes, the K_A, and the ability of each mAb to inhibit enzyme activity of native NASE were determined. All mAb had κ-L chains, seven were IgG1, two were IgG2b, one IgG2a and, one IgA (Table I). mAb-17 did not react well with any of the isoantisera even after multiple rounds of subcloning. Sequence analysis of the L chain mRNA by polymerase chain reaction (data not shown) clearly shows a single species of L chain strongly supporting the clonality of the mAb-17 hybridoma. Sequence studies of the mAb-17 H-chain are currently underway.

The binding constant of each mAb was estimated by the ELISA-based method of Friquet et al. (16) as modified by Stevens (17). Eight of the 12 mAb were of high affinity with K_A estimated as 1×10^9 M^{-1} or more (Table I) whereas that of three others was estimated as less than 10^8 M^{-1}. The binding constant for the IgA mAb was not determined.

Four of 10 mAb blocked hydrolysis of DNA by native NASE (Fig. 3). Three of the four (mAb-19, mAb-22, and mAb-25) bind to the same region of NASE (see Figs. 1 and 2). In fact, mAb-19 and mAb-25 belong to the same complementation group and mAb-22 binds to an "overlapping" epitope. The fourth mAb (mAb-1) inhibits enzyme activity, albeit at a higher concentration (consistent with its lower binding constant) and is directed to a region spatially distinct from the other three inhibitory antibodies (Fig. 2). The other mAb had little or no effect on activity even when added at very high concentrations. Data presented below and elsewhere (8) strongly suggest

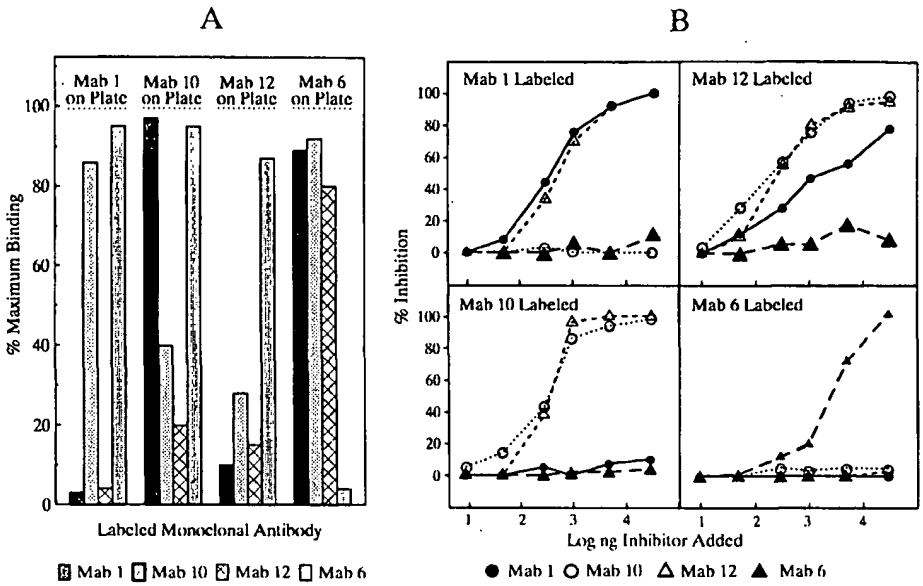

Figure 1. Complementation and competitive inhibition analysis of the binding of monoclonal antibodies to NASE. *A*, Complementation assay: NASE was added to wells of a microtiter plate that had been previously coated with a mAb. The binding of a second, HRPO-conjugated mAb to the trapped NASE was monitored by ELISA. The results are presented as percent maximum binding with 100% equal to that achieved by the excess labeled antibody in an assay where binding was not inhibited. *B*, Competitive inhibition assay: The ability of increasing amounts of an unlabeled mAb to inhibit binding to NASE (coated onto wells of a microtiter plate) of a constant amount of HRPO-conjugated mAb was monitored by ELISA. The results are presented as percent inhibition of binding of the same amount of labeled antibody in the absence of inhibitor.

TABLE I

Association constants and isotypes of anti-nuclease mAb

mAb[a]	Isotype[b]	K_A (M^{-1})[c]	mAb	Isotype	K_A (M^{-1})
1	IgG1	1×10^9	19	IgA	ND
6	IgG2b	$<10^8$	21	IgG2a	$<10^8$
10	IgG1	10^{10}	22	IgG1	10^{10}
12	IgG1	10^{10}	23	IgG2b	2×10^9
17	ND	1×10^9	24	IgG1	$<10^8$
18	IgG1	7×10^9	25	IgG1	10^{10}

[a] mAb-1, mAb-6, mAb-10, and mAb-12 are derived from fusion with CAF_1 spleen cells. The others are derived from fusion with BALB/c spleen cells.

[b] All have κ L chains.

[c] $K_A = 10^{10}$ means affinity too high to measure by ELISA.

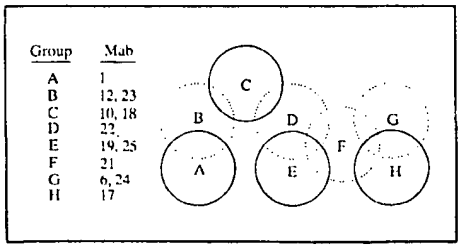

Figure 2. Complementation groups of anti-NASE mAb. Diagramatic representation of the relative positions of the epitopes detected by the anti-NASE mAb. Based on results presented in Figure 1.

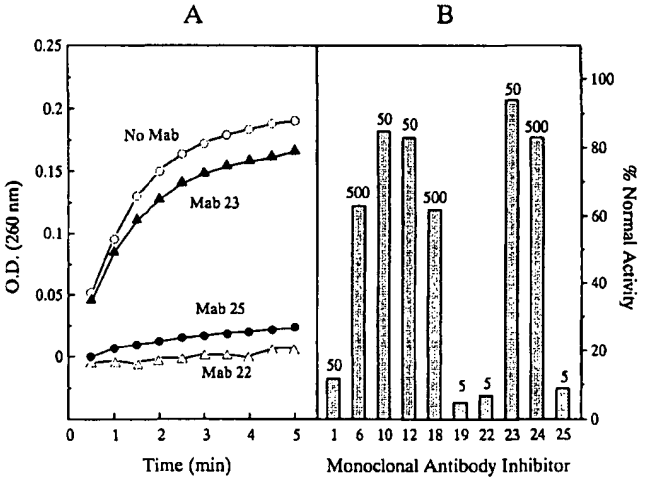

Figure 3. Effect of monoclonal antibody binding on enzyme activity. *A*, Kinetics of nuclease activity in the presence and absence of anti-NASE mAb. Enzyme was added to a solution containing antibody and DNA substrate at t = 0 and the hydrolysis of DNA monitored at 260 nm. *B*, Enzyme activity was determined in the presence of various anti-NASE mAb. The results are presented as percent of the activity in the absence of mAb. The amount of antibody added, in μg, is indicated above each *bar*.

that each antibody inhibits activity by directly blocking substrate binding rather than by inducing conformational changes when binding at sites distal to the enzyme catalytic site.

Epitope mapping using variant NASE molecules. Several variant NASE molecules were produced by mutagenesis of the cloned NASE coding sequence. These variant NASE molecules were used in a direct-binding ELISA assay in an attempt to localize one or more epitopes defined by the panel of mAb described above. The results of data compiled from several such assays are summarized in Table II. The substitutions shown at five residues (residues 49, 53, 63, 110 and 116) had little or no effect on binding by any of the nine antibodies used. In contrast, substitutions at position 46, 127, and 133 had major effects on the binding of one or more antibodies. Substitution of glutamine for lysine at position 127 (the K127Q variant; Table II) had a major effect on the binding of six of nine antibodies assayed. The broad effect of this substitution has not been investigated further but it is interesting to note that the six mAb affected bind to a cluster of "overlapping" epitopes (Fig. 2).

TABLE II

Reactivity of NASE variants with anti-NASE mAb[a]

Variant	mAb								
	1	10	12	17	18	19	22	23	25
H46R	100	100	100	100	100	100	100	100	0
K49N	90	86	85	90	100	85	100	80	70
K53N	87	98	96	ND	96	ND	ND	85	ND
K63N	100	100	100	ND	ND	ND	ND	ND	ND
K110A	85	100	100	100	100	100	100	80	100
K116A	100	100	100	90	100	100	100	100	100
K116D	100	100	100	100	100	100	100	100	100
K127Q	40	35	30	100	10	100	25	25	75
K133T	9	69	62	100	100	100	100	100	100

[a] Results are presented as percent of wild-type reaction, and are compiled from several experiments.

The other two variant molecules (H46R and K133T) each affected the binding of only a single mAb (Table II). Thus, as a first approximation, the epitopes defined by mAb-1 and mAb-25 are localized in the region of the surface residues K133 and H46, respectively.

DISCUSSION

We have analyzed the effect of amino acid substitutions on antibody binding as a method of identifying which residues of the Ag are involved in interaction with antibody. We chose NASE as a model Ag for several reasons. First, the structure of NASE is known to high resolution (19). Second, the immunology of NASE has been addressed to a limited extent (20), supplying background information for our studies. Third and more importantly, the availability of the cloned gene in an expression vector made NASE appropriate for structure-function analysis by site-directed mutagenesis. We began our analysis of the antigenic surface of NASE by generating and characterizing a panel of anti-NASE mAb.

Twelve anti-NASE mAb were selected from a large panel of anti-NASE hybridomas and characterized for use in Ag structure studies. The mAb were placed in eight complementation groups based on their distinct binding patterns, and together define a series of eight overlapping epitopes. The complementation assay that we used determines the ability of a labeled mAb to bind to Ag trapped by another mAb immobilized on the assay plate. It is assumed that any mAb that does not bind to the trapped Ag recognizes an epitope either identical to, overlapping with, or very nearby (so as to be sterically hindered from binding) the epitope recognized by the immobilized mAb. The three possibilities listed cannot be distinguished by this or any other competition assay. Therefore, we will use the term "overlapping epitope" to describe either a true overlapping epitope (shares contact residues with a nearby epitope) or a nearby epitope that does not share contact residues but that is sufficiently close so that binding of both antibodies is not possible. A recent report from Smith-Gill et al. (21) suggests that antibodies that show competitive inhibition truly overlap and for an antigen with a relatively small radius of curvature (such as NASE and lysozyme), steric hinderance is unlikely to be the cause of "competitive exclusion."

Considering the water accessible surface area of NASE (7840 A^2) (22) and an average epitope area (770A^2) (23), we estimate that the epitopes defined by the entire panel of antibodies covers a large portion of the NASE surface. This is consistent with the conclusion that the entire surface of a protein is immunogenic as proposed in the multideterminant-regulatory hypothesis (24). Four of the complementation groups have multiple members, providing reagents for analyzing fine specificity differences between antibodies that may recognize the same or very similar epitopes. The panel also contains a variety of isotypes, IgG1, IgG2a, IgG2b, and IgA, and most antibodies are of high affinity, with the majority having affinity constants of 10^9 M^{-1} or more. The method used for determining affinity constants is limited by the conditions and parameters of the ELISA assay. Thus these are conservative estimates and the K_A of several of the mAb may be more than 1×10^{10} M^{-1}.

Four mAb blocked the enzyme activity of NASE. mAb-25 maps to H46 located at the edge of the enzyme active site consistent with the ability of mAb-25 to inhibit enzyme activity. Interestingly, mAb-19, which also blocks enzyme activity, belongs to the same complementation group as mAb-25 (Table I; Fig. 2) but was unaffected by the H46R substitution. This suggests that mAb-19 and mAb-25, if they do react with the same epitope, have distinct differences in fine specificity. mAb-22, which also blocks enzymatic activity, was shown to overlap with this group. The fourth mAb (mAb-1) is directed to another region of NASE that includes the K133 residue. K133 is located at the top of a shallow groove a significant distance away. It has been suggested that residues in the vicinity of K133, although not part of the catalytic site of NASE, do participate in binding of the large m.w. DNA substrate (19). The location of the critical residues that define the epitopes for mAb-25 and mAb-1, H46 and K133 respectively, suggest that these antibodies block binding of the large DNA substrate.

We used a limited panel of single amino acid NASE variants, generated by site-directed mutagenesis, to screen these mAb in an assay designed after traditional mapping experiments using species variants. The premise of these studies is that antibody binding is sensitive to amino acid changes when these changes reside in the epitope recognized by that antibody. Using only nine single amino acid variants of NASE, we were able to map the general location of the epitopes recognized by two of nine anti-NASE mAb. These two epitopes do not overlap each other because the mAb that define them, mAb-1 and mAb-25, belong to different complementation groups. An important assumption, in this analysis, is that the single amino acid variants of NASE generate only local effects and do not cause long range structural alterations that indirectly influence antibody binding. As evidence that surface substitutions do not cause long range effects, those variants that showed reduced binding to one antibody had no effect on binding by the other antibodies. The exception was the K127Q substitution, which disrupted binding of several antibodies. However, the structural alterations in this variant are relatively local since other antibodies (mAb-17, mAb-19, and mAb-25) bound K127Q with wild-type reactivity and those antibodies with reduced binding form a cluster of antigenic regions on NASE. Further characterization of some of these and other variant NASE molecules is described in the accompanying report (8).

We have thus provided an initial description of an Ag-antibody system for which the Ag lacks disulfide bonds, for which the crystal structure is known to high resolution, and for which the coding sequences have been cloned to allow easy manipulation of the structure of the protein Ag. Using this system we have described here the production and characterization of a panel of mAb that together define a series of eight overlapping epitopes on NASE. Using site-directed mutagenesis we have mapped epitopes recognized by two of these mAb on the surface of NASE. These results and those presented in the accompanying report (8), which presents a more detailed analysis of one of these two epitopes, clearly demonstrate the efficacy of site-directed mutagenesis in the study of protein antigenicity.

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The Antigenic Structure of Bovine Serum Albumin

EVIDENCE FOR MULTIPLE, DIFFERENT, DOMAIN-SPECIFIC ANTIGENIC DETERMINANTS*

(Received for publication, November 7, 1977)

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Using antiserum to native bovine serum albumin and antigenically active fragments of the protein, we have isolated antibodies directed to each of the three domains and to several subdomains of the albumin molecule. Using albumin and these fragments as inhibitors of the reaction between ^{125}I-albumin and any given antibody population, we have demonstrated that: (*a*) each domain of albumin is antigenically distinct from each of the other domains; (*b*) each domain possesses a minimum of two different antigenic determinants; and (*c*) the entire albumin molecule possesses a minimum of six different, nonrepeating, antigenic determinants.

Recent studies (1-3) from our laboratory have demonstrated the efficacy of using immunochemical methods to study the refolding of complex protein molecules. Using antisera to native bovine serum albumin and antigenically active fragments of the albumin, we have isolated antibodies directed against each of the domains and several subdomains of the albumin molecule. These antibodies were then used to investigate the refolding of denatured albumin to its native antigenic form. These studies have demonstrated that: (*a*) albumin is composed of three independently refolding domains (2); (*b*) adjacent domains have a restrictive effect on the folding of each given domain (2); (*c*) each independently refolding domain basically follows the same sequence of refolding, *i.e.* the COOH terminus of each domain folds more rapidly than the NH_2-terminal portion (3); and (*d*) antibody may be used as a probe for possible nucleation sites.[1]

The significance of these observations requires that each population of antibody isolated from the fragment immunoadsorbent columns is specific for the region represented by that fragment. The antigenic activity of the fragments used previously (1-3) suggested that albumin is composed of multiple, different, antigenic determinants since any given fragment would only partially inhibit the reaction between antibody and albumin. Furthermore, the maximal extent of inhibition observed was proportional to the fragment size, as would be expected if albumin did possess multiple, different, antigenic determinants. These results are in agreement with a large body of evidence in previous reports on the antigenic properties of albumins (4-18).

The data presented in this paper represent a full immunochemical description of each of the antibody populations previously used. The results demonstrate that each domain of serum albumin is antigenically distinct from each of the other domains; that each domain possesses a minimum of two different antigenic determinants; and that the entire albumin molecule possesses a minimum of six different, nonrepeating, antigenic determinants.

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[1] L. G. Chavez, Jr., and D. C. Benjamin (1978) *J. Biol. Chem.* **253**, 8081-8086.

EXPERIMENTAL PROCEDURES

Materials

Crystalline bovine serum albumin was obtained from Armour; carrier and reductant-free sodium [^{125}I]iodide was obtained from Amersham/Searle. Bovine albumin was trace-labeled according to the method of McConahey and Dixon (19).

Methods

The following methods have been previously described in detail (1-3): immunization of rabbits with albumin, preparation and use of immunoadsorbents, the cleavage method and isolation procedures used to obtain each of the antigenically active fragments of albumin, and the chemical characterization of each of the fragments used.

Inhibition Assay—The assay was as previously used in our refolding studies (1-3) and is a modification of the Farr procedure (20). The following were added to each assay tube in the order given: 100 μl of ^{125}I-albumin (50 ng); 100 μl of inhibitor (albumin or fragment in varying amounts); 200 μl of a 1:4 dilution of normal rabbit serum as carrier protein; and 100 μl of a given antibody solution (previously determined to be sufficient to bind 50% of the ^{125}I-albumin in the absence of any inhibitor). All dilutions of ^{125}I-albumin, inhibitor, or antibody were made in phosphate-buffered saline ($NaCl/P_i$) containing 1% NRS.[2] The contents of each tube were mixed, incubated at room temperature for 30 min, and then cooled to 0°C in an ice bath. Saturated ammonium sulfate (500 μl) was then added, the contents of each tube mixed, and let stand at 0°C for 30 min. The precipitate was sedimented at 2000 rpm for 30 min at 4°C in an International PR-6 refrigerated centrifuge. The supernatant was decanted and the precipitate washed twice with ice-cold 50% saturated ammonium sulfate in $NaCl/P_i$. ^{125}I-albumin in each precipitate was determined in a Beckman Biogamma II γ-counter. Controls included tubes without inhibitor (binding control) and tubes without antibody (nonspecific precipitation control). Triplicate assays were carried out at each inhibitor and control concentration. The per cent inhibition was calculated as follows for each inhibitor concentration used:

$$\% \text{ inhibition} = \frac{A - B}{A} \times 100$$

where A is the per cent precipitation in the absence of inhibitor (binding control) and B is the per cent precipitation in the presence of inhibitor. The results are plotted as the per cent inhibition *versus*

[2] The abbreviation used is: NRS, normal rabbit serum.

the molar ratio of peptide:^{125}I-albumin for each concentration of inhibitory peptide used.

Nomenclature—Fig. 1 shows the domains and disulfide loop structure of bovine albumin as proposed by Brown (21). This schematic also shows the position of each fragment within the sequence of albumin and the cleavage method used to produce each fragment.

Fig. 2 shows the sequence of immunoadsorbents used to fractionate the anti-albumin into subpopulations of antibody. Figs. 1 and 2 also show the nomenclature used to describe each antibody population and each fragment used in these studies. The antibody nomenclature is such that it describes the portion of the intact molecule to which the antibody is directed, *e.g.* antibody directed to each of the three domains of albumin are anti-DI, anti-DII, and anti-DIII. Antibody to each subregion of a given domain is noted as being directed either to the COOH-terminal portion (*e.g.* anti-DI-C) or to the NH_2-terminal portion (*e.g.* anti-DI-N) of each domain. The amino acid residues, within the linear sequence of albumin, to which the antibody is directed are given in parentheses, *e.g.* antibody directed toward the first domain is given as anti-DI (N1–183); antibody to the COOH-terminal portion of the first domain is given as anti-DI-C (N115–183); and antibody to the NH_2-terminal portion of the first domain is given as anti-DI-N (N1–114). The antibody populations used in this study are double underlined in Fig. 2.

Each fragment is noted by the method used for cleavage and the amino acid residue numbers in the fragment, *e.g.* fragment $T_{377-581}$ (representing domain III) was produced by limited cleavage with trypsin and includes residue numbers N377-581; fragment $CNBr_{1-183}$ (representing domain I) was produced by cleavage with cyanogen bromide and includes residues N1–183; and fragment $P_{504-581}$ (representing the COOH-terminal portion of domain III) was produced by limited cleavage with pepsin and includes residues N504–581.

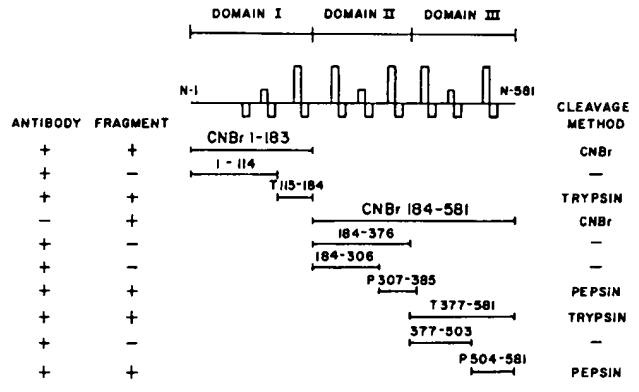


FIG. 1. Schematic representation of the domain structure of albumin, the position of the disulfide loops, and the antigenically active fragments of the molecule that have been isolated. Included in the figure are the cleavage methods used to obtain the fragments plus the regions to which the restricted populations of antibody used in these experiments are directed.

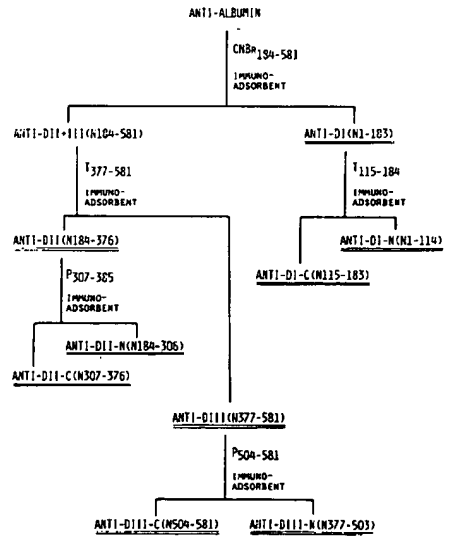


FIG. 2. Schematic representation of the sequence of immunoadsorbents used to fractionate anti-bovine albumin into subpopulations directed against the domains and several subdomains of albumin.

RESULTS

Inhibition of the Reaction of Whole Anti-albumin and ^{125}I-albumin—The inhibitors used in these experiments were: (*a*) intact native albumin; (*b*) fragment $CNBr_{1-183}$ (domain I); (*c*)

TABLE I

Specificity of antibody directed to the subdomains of bovine serum albumin

Antibody[a]	Inhibitor[b]	Maximum[c] inhibition	Molar ratio[d] at 50% maximum inhibition
Anti-albumin	Albumin	100	1.07
	$CNBr_{1-183}$	32	12.00
	$T_{377-581}$	44	2.40
	$CNBr_{184-581}$	72	6.01
	$CNBr_{1-183}$ + $CNBr_{184-581}$	100	8.90
Anti-DI (N1–183)	Albumin	100	1.00
	$CNBr_{1-183}$	100	4.50
	$T_{377-581}$	0	
	$CNBr_{184-581}$	0	
Anti-DI-C (N115–183)	Albumin	100	0.97
	$CNBr_{1-183}$	97	5.00
	$T_{115-184}$	81	90.00
Anti-DI-N (N1–114)	Albumin	100	1.10
	$CNBr_{1-183}$	98	5.40
	$T_{115-184}$	4	
Anti-DII (N184–376)	Albumin	100	1.08
	$CNBr_{184-581}$	96	7.00
	$CNBr_{1-183}$	6	
	$T_{377-581}$	3	
Anti-DII-C (N307–376)	Albumin	100	1.00
	$CNBr_{184-581}$	98	7.20
	$P_{307-385}$	98	84.00
	$T_{377-581}$	0	
Anti-DII-N (N184–306)	Albumin	100	0.97
	$CNBr_{184-581}$	95	5.70
	$P_{307-385}$	7	
	$T_{377-581}$	2	
Anti-DIII (N377–581)	Albumin	100	1.14
	$T_{377-581}$	100	2.40
	$CNBr_{1-183}$	7	
Anti-DIII-C (N504–581)	Albumin	100	1.10
	$T_{377-581}$	98	4.70
	$P_{504-581}$	90	21.00
	$P_{307-385}$	0	
	$T_{115-184}$	0	
Anti-DIII-N (N377–503)	Albumin	100	1.17
	$T_{377-581}$	100	2.90
	$P_{504-581}$	6	

[a] Antibody solution used in the inhibition assay. Numbers in parentheses represent the amino acid position number in the sequence of albumin against which the antibody is specific. See text for full nomenclature description.

[b] Peptides used in the inhibition assay. Numbers represent the position of the peptide in the sequence of albumin. See text for full nomenclature description.

[c] Maximum inhibition observed at the highest concentration of inhibitor used.

[d] Molar ratio, of peptide to ^{125}I-albumin, in the assay at which 50% maximum inhibition was observed.

fragment $T_{377-581}$ (domain III); (*d*) fragment $CNBr_{184-581}$ (domains II + III); and (*e*) an equimolar mixture of fragments $CNBr_{1-183}$ and $CNBr_{184-581}$. The results shown in Fig. 3 and Table I demonstrate that only intact albumin and the equimolar mixture of fragments $CNBr_{1-183}$ and $CNBr_{184-581}$ will inhibit 100%. Each of the fragments used alone gives incomplete inhibition. The sum of the maximum inhibitions given by fragments $CNBr_{1-183}$ and $CNBr_{184-581}$ (together representing all three domains) is 104% and thus accounts for all the antigenic activity of albumin. Indeed, addition of an equimolar mixture of these two fragments results in 100% inhibition (Table I) (14, 22). To date we have not been able to isolate a fragment representing domain II alone. However, since fragment $T_{377-581}$ represents domain III and fragment $CNBr_{184-581}$ represents domains II + III (Fig. 1), the difference between the maximum inhibition given by each of these fragments should represent the fraction of the total anti-albumin that is specific for domain II, *i.e.* 72% − 44% = 28%. A similar result is obtained if one subtracts the maximum inhibitions given for fragments $CNBr_{1-183}$ (domain I) and $T_{377-581}$ (domain III) from that given for intact albumin (domains I + II + III), *i.e.* 100% − (32% + 44%) = 24%. The fraction of the total anti-albumin reactive with any given domain varied slightly from antiserum to antiserum, as would be expected, yet was fairly evenly distributed.

The molar ratio of peptide:albumin required for 50% maximum inhibition is indicative of the relative integrity of the antigenic determinants on each fragment. The ratios observed (Table I) indicated that each fragment has retained a high degree of native antigenic conformation. The specificity of each antibody population for native structure has been previously demonstrated with reduced albumin and reduced fragments (1-3), and in addition none of the antibody populations used in this paper was reactive with reduced and carboxymethylated albumin or fragments.

Inhibition of the Reaction between ^{125}I-Albumin and Antibodies to Each Domain—The specificity of antibody directed against each of the three domains was determined by using albumin and various fragments as inhibitors. The reaction between ^{125}I-albumin and anti-DI (N1-183) was inhibited 100% by both albumin and by fragment $CNBr_{1-183}$ (Fig. 4*A*). The molar ratios at 50% maximum inhibition were 1.0 and 4.5, respectively (Table I). Fragments representing other domains showed no inhibition even at very high molar ratios. Thus, antibody not absorbed by columns bearing fragment $CNBr_{184-581}$ (negative selection) was specific for the region of albumin representing domain I. Identical results were ob-

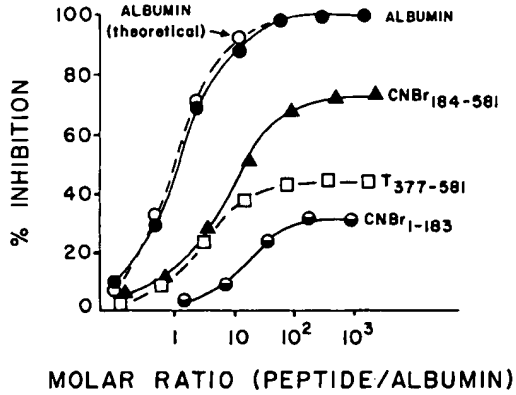


FIG. 3. Inhibition of the reaction between anti-albumin and ^{125}I-albumin. Reaction tubes contained 50 ng of ^{125}I-albumin and anti-albumin sufficient to bind 50% of the ^{125}I-albumin and varying amounts of albumin or fragments of albumin as inhibitory peptides. Inhibitors: ●——●, fragment $CNBr_{1-183}$ (domain I); □- - -□, fragment $T_{377-581}$ (domain III); ▲——▲, fragment $CNBr_{184-581}$ (domains II + III); ●——●, albumin (domains I + II + III); ○- - -○, theoretical inhibition curve calculated from the amount of albumin known to be added as inhibitor. All assays were carried out in triplicate. Standard error between triplicates never exceeds that represented by the width or diameter of the symbol used.

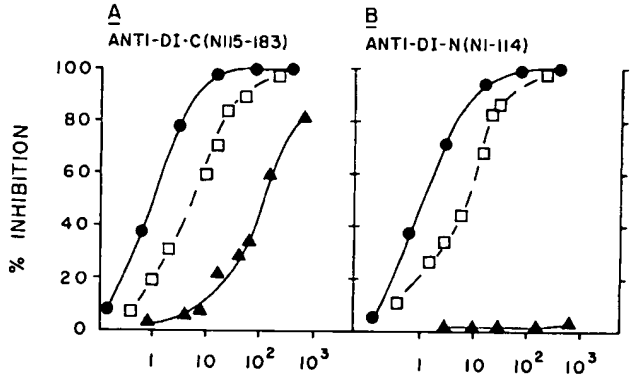
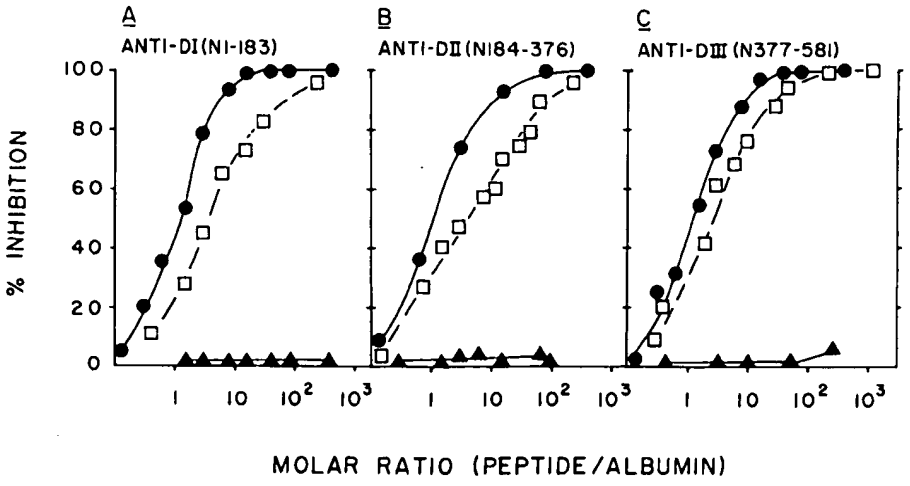


FIG. 5. Inhibition of the reaction between ^{125}I-albumin and antibodies to subregions of domain I. Conditions were the same as Fig. 3 except that the subpopulations of antibody indicated were used at concentrations sufficient to bind 50% of the ^{125}I-albumin. *A*, anti-DI-C (N115-183). Inhibitors: ●——●, albumin; □- - -□, fragment $CNBr_{1-183}$; ▲——▲, fragment $T_{115-184}$. *B*, anti-DI-N (N1-114). Inhibitors: ●——●, albumin; □- - -□, fragment $CNBr_{1-183}$; ▲——▲, fragment $T_{115-184}$.

FIG. 4. Inhibition of the reaction between ^{125}I-albumin and antibodies to each domain of albumin. Conditions were as described in Fig. 3 except that the subpopulations of antibody indicated were present at concentrations sufficient to bind 50% of the ^{125}I-albumin. *A*, anti-DI (N1-183). Inhibitors: ●——●, albumin (domains I + II + III); □- - -□, fragment $CNBr_{1-183}$ (domain I); ▲——▲, fragment $CNBR_{184-581}$ (domains II + III) or fragment $T_{377-581}$ (domain III). *B*, anti-DII (N184-376). Inhibitors: ●——●, albumin; □- - -□, fragment $CNBr_{184-581}$ (domains II + III); ▲——▲, fragment $T_{377-581}$ or fragment $CNBr_{1-183}$. *C*, anti-DIII (N377-581). Inhibitors: ●——●, albumin; □- - -□, fragment $T_{377-581}$; ▲——▲, fragment $CNBr_{1-183}$.



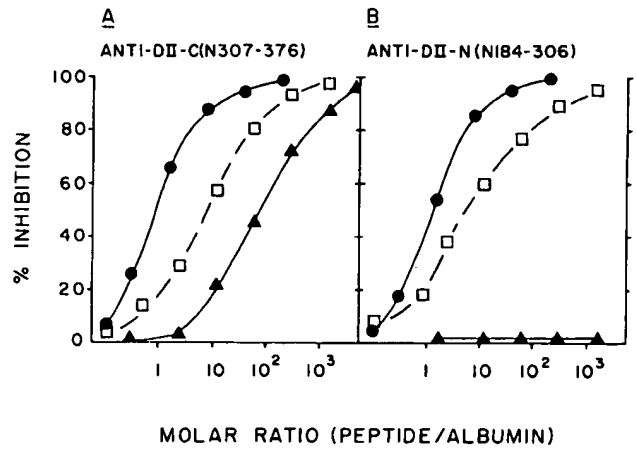


FIG. 6. Inhibition of the reaction between ^{125}I-albumin and antibodies to subregions of domain II. Conditions were the same as Fig. 3 except that the subpopulations of antibody indicated were used at concentrations sufficient to bind 50% of the ^{125}I-albumin. *A*, anti-DII-C (N307-376). Inhibitors: ●——●, albumin; □- - -□, fragment $CNBr_{184-581}$; ▲——▲, fragment $P_{307-385}$. *B*, anti-DII-N (N184-306). Inhibitors: ●——●, albumin; □- - -□, fragment $CNBr_{184-581}$; ▲——▲, fragment $P_{307-385}$.

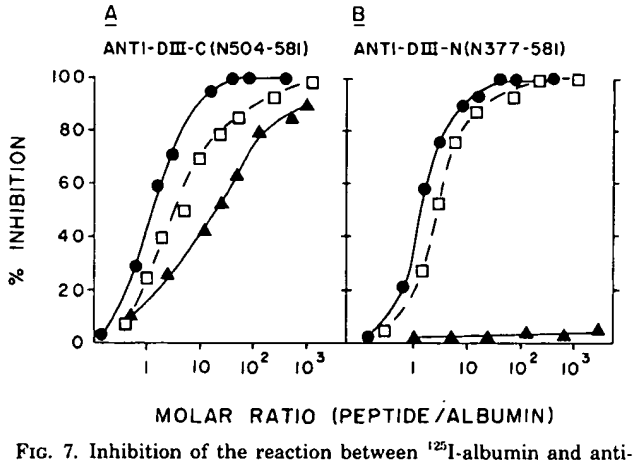


FIG. 7. Inhibition of the reaction between ^{125}I-albumin and antibodies to subregions of domain III. Conditions were the same as Fig. 3 except that the subpopulations of antibody indicated were used at concentrations sufficient to bind 50% of the ^{125}I-albumin. *A*, anti-DIII-C (N504-581). Inhibitors: ●——●, albumin; □- - -□, fragment $T_{377-581}$; ▲——▲, fragment $P_{504-581}$. *B*, anti-DIII-N (N377-503). Inhibitors: ●——●, albumin; □- - -□, fragment $T_{377-581}$; ▲——▲, fragment $P_{504-581}$.

tained with antibody eluted from fragment $CNBr_{1-183}$ immunoadsorbents (positive selection).

Similar assays were performed with antibody directed against domains II and III (Fig. 4, *B* and *C*, respectively, and Table I) with identical results, *i.e.* these antibodies were reactive only with albumin and those fragments containing the correct domain.

Inhibition of the Reaction between ^{125}I-Albumin and Antibodies to Subregions of Each Domain—Anti-DI (N1-183) was further fractionated into two antibody populations (Figs. 1 and 2) directed against the COOH-terminal portion and the NH_2-terminal portion of domain I using an immunoadsorbent containing fragment $T_{115-184}$. These antibody populations were termed anti-DI-C (N115-183) and anti-DI-N (N1-114), respectively. The specificity of these two antibody populations was determined using albumin and various fragments as inhibitors. The results presented in Fig. 5*A* demonstrate that anti-DI-C (N115-183) was inhibited 100% by albumin, by fragment $CNBr_{1-183}$, and by fragment $T_{115-184}$ with molar ratios at 50% inhibition of 0.97, 5.0, and 90.0, respectively (Table I). The relatively high molar ratio for fragment $T_{115-184}$ demonstrates that this smaller fragment had undergone more conformational changes than the same region in fragment $CNBr_{1-183}$. No significant inhibition was observed with non-domain I fragments, *i.e.* fragments $CNBr_{184-581}$, $T_{377-581}$, $P_{307-385}$, or $P_{504-581}$.

Fig. 5*B* shows that anti-DI-N (N1-114) was inhibited 100% by albumin and by fragment $CNBr_{1-183}$ with 50% inhibition molar ratios of 1.10 and 5.4, respectively (Table I), but not by fragment $T_{115-184}$ or any non-domain I fragments. Thus, domain I of albumin possesses at least two different antigenic determinants, one residing within the NH_2-terminal portion and one within the COOH-terminal region.

Figs. 6 and 7 and Table I present the results of identical determinations of the specificity of antibody directed against subregions of domains II and III, respectively. Thus, each domain of albumin possesses two different antigenic determinants unique to that domain for a minimum of six nonrelated determinants on the intact protein. No cross-reacting determinants between domains could be demonstrated with this, or any other, antiserum.

DISCUSSION

The results presented in this paper demonstrate a minimum of six different, nonrepeating, antigenic determinants on bovine serum albumin with a minimum of two antigenic determinants unique to each of the three domains of the molecule. No evidence was found for determinants common to the three domains.

Inhibition of the reaction between antinative albumin and ^{125}I-albumin by fragments representing one or more domains was never complete even at a 1000-fold molar excess of the fragment. In contrast, inhibition with the native protein (containing all three domains) was 100% and was essentially identical with that predicted based on the quantity of inhibitor albumin added (Fig. 3). The maximum inhibition observed (or calculated) for the regions representing domains I, II, and III was 32%, 24 to 28%, and 44%, respectively. Thus, the amount of antibody reactive with each domain was fairly evenly distributed. This would suggest that each domain possesses an equal number of antigenic determinants. Based on what is known of the antigenic structure of sperm whale myoglobin (23) and assuming the number of antigenic determinants on a protein molecule is directly proportional to molecular weight, one would predict six to seven determinants per domain for albumin and a total of 18 to 21 on the entire molecule.

The fact that no fragment would give complete inhibition of the reaction between albumin and whole anti-albumin suggests that each fragment possessed some, but not all, of the antigenic determinants of the protein (Fig. 3) (2, 14, 22). We have previously shown that the maximum extents of inhibition by two nonrelated fragments of albumin are additive (14, 22). These results were confirmed in the present study (Table I) and are similar to results obtained with fragments of sperm whale myoglobin bearing different antigenic determinants (24).

Anti-albumin was fractionated into subpopulations of antibody using immunoadsorbents bearing fragments of the molecule (Fig. 2). Each subpopulation was shown to be specific for a given region in that its reaction with ^{125}I-albumin was inhibited only by albumin or by fragments containing that region of albumin. Whereas the reaction between ^{125}I-albumin

and anti-albumin was only partially inhibited by any given fragment (Fig. 3), the reaction between ^{125}I-albumin and antibody to any given region was completely inhibited by a fragment containing that region but not at all by fragments not possessing that region (Figs. 4 to 7 and Table I). This can only be interpreted to mean that: (*a*) albumin and the corresponding fragment bear the same antigenic determinants with reference to a given subpopulation of antibody; and (*b*) these antigenic determinants are not found elsewhere on the albumin molecule.

The molar ratio of peptide to ^{125}I-albumin required for 50% maximum inhibition is indicative of the structural integrity of the antigenic determinants on the fragment relative to the same determinant on albumin. The fragments used in these studies were found to be relatively efficient inhibitors with molar ratios at 50% maximum inhibition ranging from 2.4 to 90.0 (Table I). The larger fragments, representing one or more domains, were the most efficient. The relatively large molar ratios of 6.0 and 12.0 for fragments $CNBr_{1-183}$ and $CNBr_{184-581}$ as compared to 2.4 for fragment $T_{377-581}$ and 1.0 for albumin itself may have been due to slight conformational changes caused by cleavage at internal methionine residues by cyanogen bromide. The larger molar ratios for the smaller fragments (Table I) indicate a greater degree of conformational change as compared to the same regions of intact albumin.

The hypothesis presented above that serum albumin contains multiple, nonrepeating, antigenic determinants is supported by evidence from a large number of studies dealing with the antigenic activity of fragments of serum albumins (4–9, 14, 17, 18, 22), antibody synthesis by single cells (15), serological cross-reactions of antibody to albumins (10, 11), and specific acquired immunological tolerance to albumins or to fragments of albumins (12, 13, 16).

In contrast to all of the above, Atassi and his colleagues (25–28) have recently suggested that bovine albumin is composed of three antigenically identical (or very similar) domains. The techniques they used, although different from those used by us, are similar to those used by Porter (4, 5), Lapresle (6, 7), Weigle (10), and by Timpl *et al.* (11), all of whom could find no evidence for repeating antigenic determinants on serum albumins. The results of Atassi and his colleagues do present some anomalies, as follows: (*a*) anti-bovine albumin would not precipitate with a fragment representing the majority of domain I (26) or domain III (27), yet antibody produced to either fragment would not only precipitate with bovine albumin but also with both fragments (28); (*b*) absorption of anti-fragment with the other fragment removed all antibody precipitating with albumin or the absorbing fragment, but not with the fragment to which the antibody was produced (26); and (*c*) multiple absorptions of anti-albumin by either fragment were required to remove all antibody (26, 27). However, recent reports from another laboratory (17, 18), using immunoadsorbent techniques similar to those used by Atassi (26–28), fully support the concept of multiple, nonrepeating determinants. These differences in results between the studies of Atassi and his colleagues (25–28) and those of others (4–18, 22) and the present study cannot yet be fully explained.

One would not necessarily expect cross-reactions between antibodies directed to structurally homologous regions of albumin. The maximum extent of identity in amino acid sequence between any two of the three domains is 25% (29). Indeed, if one determines the identity shared between all three domains it is only 19/193 residues or 10%. Of these 19 identical residues, 8 are cysteine residues involved in disulfide bonds and apparently conserved in all mammalian albumins and possibly in avian albumins (21, 29, 30). If one compares the amino acid sequences of bovine and human serum albumin (21, 30), it is seen that of the 19 residues identical between the three domains of bovine albumin 18/19 are also found in human albumin. These residues, therefore, are probably required for domain structure and function and are probably highly conserved throughout evolution. This is even more striking if one considers that human and bovine albumins cross-react only to the extent of 15% (10, 11), yet are approximately 80% identical in sequence (29).

Antibody produced to albumin in rabbits would be directed to structures not shared between bovine and rabbit albumins. If rabbit albumin shows a high degree of sequence homology with bovine albumin, as does the human protein, and if the residues that are identical between domains of albumin are also found in rabbit albumin, as they are in human albumin, then one would not expect the rabbit immune system to see these regions as antigenic determinants. Although we cannot assume that identity in amino acid sequence is required for antigenic cross-reactivity, we can assume that mammalian antibody produced against mammalian proteins will be primarily directed toward structures that are quite different (and not conserved) between the antigen and the same protein molecule in the responding animal. Thus, only in those cases where the antibody to albumin is produced in very distantly related species (*i.e.* avian or reptilian) or in individuals genetically deficient and thus not immunologically tolerant to their own species albumin (*i.e.* analbuminemia in humans) would we predict the production of antibody which recognizes similar structures in each of the three domains of bovine albumin.

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